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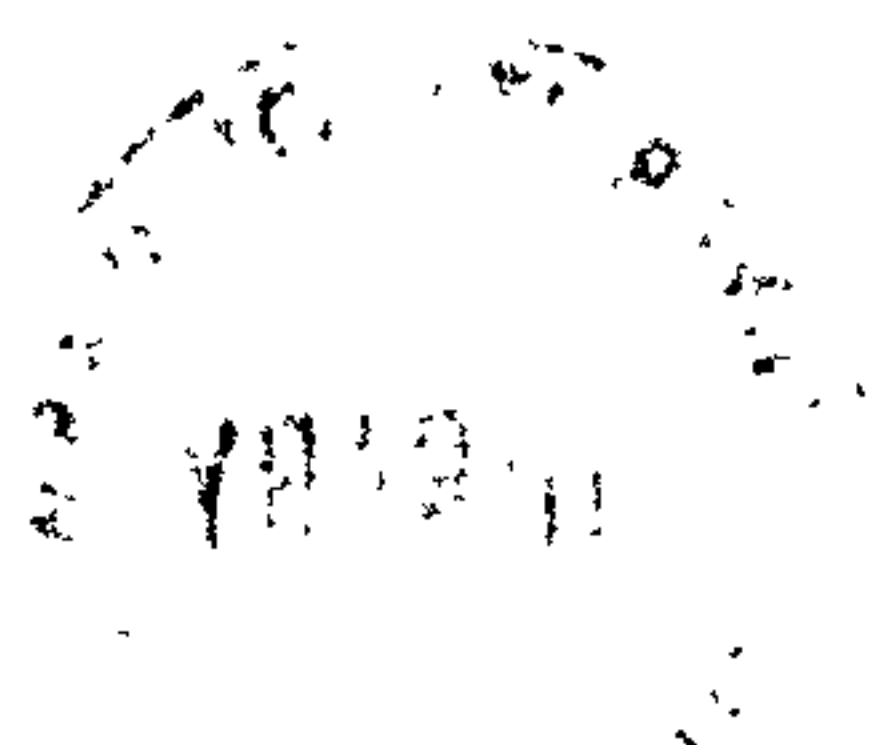
ATTACHMENT OF FRESHWATER BACTERIA TO SOLID SURFACES

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nothing which is the outcome of work done in collaboration

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Summary

The initial stages of the permanent attachment of selected freshwater bacteria, particularly Pseudomonas fluorescens, Enterobacter cloacae, a Chromobacterium sp and a Flexibacter sp, to a hydrophobic and a relatively hydrophilic polystyrene surface was investigated.

Changes in the nutrient conditions and growth rates of the bacteria caused differences in cell surface characteristics, measured by hydrophobic and electrostatic interaction chromatography and saline contact angle measurements on lawns of cells, and changed attachment levels to the two substrata in both buffer and nutrient media. The effects were different for each species, varying independently with surface, and were, largely, related to changes in predicted physico-chemical interactions. However, the Flexibacter sp, a gliding bacterium, showed an increase in attachment with increased growth rate, i.e. metabolic activity, possibly indicating an active attachment process.

Peaks in the attachment of E. cloacae, P. fluorescens and the Chromobacterium sp to both substrata occurred between pH 6 and 7.5 and 20°C and 25°C, while the Flexibacter sp decreased attachment with increasing pH and temperature. These changes in attachment for each species were related to physico-chemical rather than physiological effects.

Varying the electrolyte concentration and valency of the attachment solution showed that permanent attachment was independent of diffuse electrical double layer thickness. Similarly, attachment levels could not be predicted thermodynamically using liquid, solid and cell surface tensions, indicating the involvement of electrostatic interactions in the attachment of these species. Estimates of the hydrophobicity of the cell surfaces, by hydrophobic interaction chromatography, showed that hydrophobic interactions did not dominate in bacterial attachment. Thus, attachment of these four species was a multifactorial interaction involving a range of physico-chemical interactions.

The attachment of individual species was also found to be affected by the presence of other bacterial species. Thus in natural aquatic systems a variety of biological, environmental and physico-chemical parameters will affect levels of bacterial attachment and hence biofilm composition and development.

CHAPTER ONE

INTRODUCTION

1.1 AIMS

This thesis has two aims: first, to determine some of the microbiological, physico-chemical and environmental factors affecting the initial stages of bacterial attachment to solid surfaces; second to determine whether bacterial species differ in the mechanisms of their attachment to those surfaces.

The organisms selected for investigation are freshwater bacteria; however, the factors affecting bacterial attachment and permanent adhesion to solid substrata may be similar for marine bacteria. Generally the discussion will be in terms of attachment in freshwater and marine habitats, though on occasions consideration of attachment in other systems, e.g. to teeth, will be advantageous, since similar forces may well act on the attaching organisms. In this Chapter it is hoped to outline in general terms features of bacterial attachment, providing a framework for the more detailed discussion in subsequent Chapters, and also to describe the development of bacterial biofilms.

1.2 SOLID/LIQUID INTERFACES AND THE SIGNIFICANCE OF MICROBIAL BIOFILMS

In natural aquatic environments a heterogenous array of solid surfaces is presented to microorganisms. The variety of surfaces is immense, ranging from rock, sand grain and artificial man-made surfaces, e.g. ship hulls and pipelines, to plant and animal surfaces. The boundary between a solid surface and the bulk liquid phase can be defined in physico-chemical terms as an interface, i.e. the boundary between two phases in a heterogenous system (Marshall, 1976). A solid/liquid interface will have significant effects on the distribution of macromolecules and ions, on the pH, aeration, nutrient availability and the presence of inhibitors in its local microenvironment. Such interfaces possess unique environmental conditions different from

both the bulk liquid phase and the solid substratum, and it is within this interfacial habitat that many bacteria attach and succeed.

Given the ubiquitous nature of bacteria it is not surprising that large bacterial populations are often found at solid/liquid interfaces, and that they represent the primary colonizers of such environments. As early as 1933, Henrici demonstrated the occurrence of bacterial attachment in water systems by immersing glass slides, for varying periods of time, staining and examining them microscopically. Since then many different microscopic techniques, e.g. epifluorescence and scanning electron microscopy, have been used by a variety of workers, to further evaluate bacterial adhesion to surfaces (Corpe, 1974a; DiSalvo & Daniels, 1975; Paerl, 1975). Sieburth (1975) made an extensive survey of marine bacterial attachment to a variety of surfaces both inanimate and biological; the result was a clear demonstration of the frequency of bacterial adsorption to surfaces - a commonplace occurrence.

The significance of bacterial attachment to solid substrata in aqueous environments should not be underestimated. The importance of this phenomenon lies not only in the role attached microorganisms play in natural habitats, but also the effect such attachment has on man-made structures.

The fact that microbial films are present on so many submerged surfaces indicates the significance of periphytic bacteria in natural aquatic ecosystems. Costerton et al. (1978) estimated that 1 million bacteria attach per cm^2 of solid surface compared with 1000 free-living bacteria per cm^3 in the aqueous phase. The attached bacteria can increase to 10^9 cells per cm^2 in polluted water. The effect of periphytic bacteria on natural ecosystems clearly must be large and Paerl (1980) emphasized the ecological significance of such films. Attached bacteria may be important in the biodegradation of organic substances since organic molecules are attracted to solid surfaces (Section 1.3, Chapter 6) and may represent an important component in

the recycling of nutritive material within the aquatic system. Fletcher (1979a) suggested that biofilms might also act as a 'microbial reservoir' with cells released from the solid surface replenishing the aqueous phase. The role played by bacterial biofilms in subsequent attachment of invertebrates has been emphasized by several workers. The microbial film has been proposed to condition favourably a solid surface and prepare it for fouling by invertebrates larvae (Wood, 1967; ZoBell & Allen, 1935). However, the presence of a bacterial community on a solid substratum has also been shown to inhibit the settlement of some larvae (Corpe, 1976). The bacterial film itself might to some extent determine subsequent fouling sequences of solid surfaces.

Microbial biofilms have been used to man's advantage. Adsorbed bacteria in trickling filters are used in the treatment of waste water. Extensive biofilms that develop in these systems are capable of inactivating sewage bacteria and decomposing organic matter (Mack et al. 1975). Recently, bacterial cells have been immobilized in a variety of different matrix substances or on a range of surfaces to allow the exploitation of their metabolic capabilities for various industrial processes (Jack & Zajic, 1977).

Bacterial films also have detrimental effects in certain man-made systems, e.g. in pipelines, on ship hulls, and in heat transfer equipment. Microbial fouling of ship and pipeline surfaces increases fluid frictional resistance, while that of heat transfer equipment means that the heat transfer resistance of the metallic structures is altered due to the insulating effect of the bacterial film (Characklis, 1980). Problems resulting from bacterial adhesion also occur in artificial culture systems, both in laboratory and industrial systems. Hence an adhesive mutant attaching to a surface might 'take-over' from a selected parent organism (Munsen & Bridges, 1964) and the majority of cells in a culture may originate from wall growth (Larson & Dimmick, 1964).

1.3 THE SEQUENCE OF EVENTS IN THE DEVELOPMENT OF A MICROBIAL BIOFILM

The primary stage in the fouling of a solid surface is not biological in nature, but rather fouling is initiated by a physico-chemical interaction. When a clean surface is immersed in an aquatic habitat the initial event is the spontaneous adsorption of organic macromolecules. This results in the formation of a conditioning film, in which glycoproteins may predominate in natural habitats (Baier, 1980a). This conditioning film has two consequences, first it alters the charge, free energy and wetting properties of the solid surface and, second, it may provide a rich nutrient source for bacteria. The nature and extent of the conditioning film will affect the adhesion of bacteria to the surface, i.e. the physico-chemical interaction (Fletcher & Loeb, 1976; Baier, 1980b) an aspect investigated in the thesis (Chapter 6).

The subsequent microbiological colonization of solid substrata shows a distinct succession strikingly similar for both marine and freshwater ecosystems. Bacteria are the initial colonizers, followed by a diverse community of more complex organisms. The metabolic capabilities of bacteria seem to be important in the succession, and it is useful at this point to define two different bacterial nutritional types. The first is zymogenous bacteria, more recently termed copiotrophs (Poindexter, 1981) which grow poorly in low nutrient conditions but are capable of a rapid response to high nutrient levels. They have low substrate affinities, i.e. high half saturation constant (K_s) and high maximum growth rate (μ_{max}). Copiotrophic bacteria are among the most common types in many habitats even though incapable of growth in poor nutrient conditions (Hirsch, 1979). Pseudomonads, Escherichia coli etc. are good examples of copiotrophic bacteria and it is members of this group which are investigated in this work. The second metabolic type is oligotrophic bacteria such as Caulobacter and Hyphomicrobium spp. These bacteria are able to grow in a low nutrient environment, typical of many aqueous habitats. They have a low μ_{max} and very efficient uptake systems, i.e. very low K_s (Hirsch,

1979). It is unnecessary to discuss the proposed differences between copiotrophs and oligotrophs further here, although, more differentiating characteristics have been suggested (Hirsch, 1979).

Copiotrophic bacteria are the pioneering colonizers of many different substrata in both marine and freshwater habitats (Marshall et al. 1971a; Corpe, 1973; Paerl, 1975; Jordan & Staley, 1976; Dempsey, 1981). Their invasion may be favoured by the presence of a rich nutritive conditioning film (Fletcher & Marshall, 1982). The firm attachment of copiotrophic bacteria to a surface occurs within a few hours of its immersion, and the film develops further through a continuing process of bacterial adsorption and bacterial growth with bacterial microcolonies coalescing to give a unified film. The dominance of either adsorption or growth in biofilm development is probably related to environmental conditions and the species of bacteria involved. Corpe (1973) indicated that the copiotrophic bacteria were predominantly Gram-negative rods with representatives from several different genera. Pseudomonas spp generally predominated in the bacterial flora at these early colonization stages (50-90% of the population) followed by pigmented Flavobacterium species (10-49%) or nonpigmented nonmotile Achromobacter species. The copiotrophic periphytic community may become enmeshed in a polymeric matrix which may represent a small or a large component of the surface biofilm (Dempsey, 1981; Jordan & Staley, 1976).

The second phase in bacterial colonization of a solid surface is the invasion by oligotrophic bacteria, such as Hyphomicrobium and Caulobacter spp, and predatory gliding bacteria such as Saprospira spp (Marshall et al. 1971a; Corpe, 1973). This occurs after 24 to 72 hours of biofilm initiation. The late arrival of oligotrophic bacteria in the succession might find its explanation in one of two factors: firstly, a conditioning of the solid substratum by the copiotrophic bacteria allowing their invasion (Corpe, 1973), but there is no evidence for this; or, secondly, the production of nutrient-deficient conditions by the copiotrophic bacteria

favouring the success of the secondary oligotrophic colonizers (Marshall, 1980).

Later stages of biofilm formation, between 4 and 5 days, after initiation, involve an increasing complexity in the type of organisms associating with the surface. Microalgae appear as part of the film (Corpe, 1973; Jordan & Staley, 1976), and a complex array of fungi, diatoms, and protozoa become associated with the biofilm and its micro-environment (Marszalek et al. 1979). Thus a complex and diverse community develops, which is competing, interdependent and represents a significant biological component of aquatic habitats.

1.4 PHYSICO-CHEMICAL INTERACTIONS IN BACTERIAL ATTACHMENT TO SOLID SURFACES

Since copiotrophic bacteria are the primary colonizers and represent an important component of the surface microflora, their mechanisms of attachment are worthy of consideration and have been investigated in this work. Generally, their adhesion to solid surface is non-specific and permanent. The non-specific permanent adhesion (Marshall, 1980) of bacteria to solid surfaces can be divided into two components: the first, the deposition of the bacteria on/or near the surface and, the second, the attachment of the bacteria to that surface. Such factors as bacterial motility, Brownian motion and hydrodynamic effects will bring bacteria into the vicinity of a solid/liquid interface. Further interaction and subsequent attachment to the solid surface will involve a range of physico-chemical interactions and biological influences.

Marshall et al. (1971b) described two types of adsorption phenomena by bacteria at a solid surface:

1) Reversible sorption. This can be described as the initial stage of permanent adhesion. It is considered to be an instantaneous attraction of the bacteria to a solid surface, with bacteria held at a finite distance from that surface. Bacteria which are reversibly sorbed exhibit

Brownian motion and, if motile, can swim away from the surface. They are easily removed from the surface by washing.

2) Irreversible sorption. This involves the permanent adhesion of a bacterium to a substratum. The organism no longer exhibits Brownian motion and cannot be removed from the surface by washing. Irreversible attachment of bacteria is considered to be caused by a physico-chemical adsorption of the bacterial cell or bacterial surface components onto the solid surface.

The fact that bacterial adsorption to solid surfaces has been described by Langmuir-type adsorption isotherms indicates the important role of physico-chemical parameters (Fletcher, 1977; Schuraga et al. 1979). However, the possible mechanisms of physico-chemical attachment involve many different phenomena. The types of physico-chemical parameters affecting adhesion can be divided into long range forces, e.g. electrostatic interactions which are considered in DLVO theory (Shaw, 1970; Section 5.2) and account for relatively non-specific interactions and short range forces, e.g. hydrophobic bonding, which are described by surface energies and occur once molecular contact between the bacterium and the surface has been made. These are more dependent on specific molecular properties of the solid surface and the bacterial surface than the long range forces. The balance of attractive and repulsive forces will determine whether bacterial attachment to a solid surface will occur. Daniels (1980) described a range of possible interactions between a bacterium and a solid surface which would result either in attraction or repulsion between the two surfaces (Table 1.1).

Irreversible attachment to a solid non-biological substratum is probably then, relatively non-specific involving a balance of physico-chemical interactions between the solid surface, bacterial surface and liquid phase which promote or inhibit bacterial adsorption. Such attachment does not necessarily involve any specialized bacterial structures,

TABLE 1.1 Forces of attraction and repulsion between microbial cells
and adsorbent surfaces

<u>Forces of attraction</u>	<u>Forces of repulsion</u>
1. Chemical bonding (hydrogen, thio, amide + ester)	1. Charge repulsion between surfaces of similar charge
2. Ion-pair formation ($-\text{NH}_3^+ \text{ --- } ^-\text{OOC}-$)	2. van der Waals forces of repulsion
3. Ion-triplet formation ($-\text{COO}^- \text{ --- } \text{Ca}^{2+} \text{ --- } ^-\text{OOC}$)	3. Steric exclusion (hindrance)
4. Interparticle bridging (poly-electrolytes)	
5. Charge fluctuations	
6. Charge mosaics	
7. Charge attraction of opposite signs	
8. Electrostatic attraction between surfaces of opposite charge	
9. Electrostatic attraction due to image forces	
10. Surface tension	
11. van der Waals forces of attraction	
12. Electromagnetic forces	

(from Daniels, 1980)

but rather is related to characteristics and features of the cell surfaces. These features and the physico-chemical phenomena influencing bacterial attachment are dealt with, in depth, in Chapters 4, 5 and 6.

1.5 THE CELL SURFACE STRUCTURE OF GRAM-NEGATIVE BACTERIA

It is useful at this point to briefly discuss the structure and components of bacterial cell walls. The discussion will be limited to Gram-negative organisms since they predominate on solid surfaces in aquatic habitats, and they represent the bacteria primarily investigated in this work.

The Gram-negative cell wall, or envelope, is a complex multilayered structure generally considered to consist of the following layers (inwards from the cell surface):

- 1) Lipopolysaccharide (LPS)
- 2) The outer membrane, a unit of three layers
- 3) A structureless zone, the periplasmic space
- 4) A thin peptidoglycan layer
- 5) The cytoplasmic membrane, a unit membrane of three layers

(Rogers et al 1980),

The outer two layers, i.e. 1 and 2 above, which potentially might be involved in attachment interactions consist of phospholipid and lipopolysaccharide moieties (Costerton et al. 1974; Rogers et al. 1980). The oligosaccharide portions of LPS molecules are predominantly associated with the outermost section of the outer membrane although some associate with the inner section of the unit membrane, the framework of which consists of proteins and phospholipids.

Recently a considerable amount of work has been done on the proteins of the outer membrane. There are a small number of major proteins, among which are the 'porins' which form non-specific channels for small molecules through the membrane and are, probably, transmembrane units (Braun, 1978). Another major protein constituent is the lipoprotein, the majority of which is free in the outer membrane though approximately one third of it appears to anchor the outer membrane to the peptidoglycan layer. Several other minor proteins act in the high affinity transport of specific molecules

and as bacteriophage and colicin receptors. Various degradative enzymes have also been found at the cell surface (Costerton et al. 1974). A large proportion of these outer membrane proteins reacted with a specific labelling reagent indicating that they were exposed to the external medium (Kamio & Nikaido, 1977). There is considerable evidence, therefore, for the exposure of cell surface proteins to the external environment.

In contrast to the accumulation of many proteins at the outside surface of Gram-negative cells, the phospholipids are largely confined to the inner section of the outer membrane. However, LPS is located almost exclusively in the outermost portion of this membrane, protruding towards the surrounding medium (Costerton et al. 1974). The structure of the LPS is complex and includes apparently unique sugar moieties. LPS comprises three major regions, i.e. an 'O' antigen polysaccharide side chain, which is linked to an oligosaccharide core and thence to a glucosamine-containing lipid. The core region consists of an oligosaccharide outer core and an inner section of L-glycerol-D-manno-heptose (abbreviated to heptose); 3-deoxy-D-mannooctulosonate (2-keto-3-deoxyoctonate, KDO); ethanolamine and phosphate. Heptose and KDO are sugar moieties unique to the LPS region.

There is a wide variation in the amount of LPS produced by bacterial species and strains. Shands (1966) showed that the LPS of Escherichia coli and Salmonella typhimurium extended as far as 150 nm from the cell. In general enteric bacterial strains possessing complete LPS, i.e. smooth strains, are a more virulent form of the organism. Rough mutants, less pathogenic, have reduced 'O' side chains or oligosaccharide cores (Ward & Berkeley, 1980). Either of the components of the inner core may be reduced, and/or the components and length of the 'O' side chain can differ.

Further regions of cellular products, extracellular polymers and capsules can be found exterior to the cell wall. These may be involved in bacterial attachment phenomena. The size of the capsule and the extent of exocellular polymer production are considerably affected by nutrient

and growth conditions (Wilkinson, 1958). These external layers are generally polysaccharide in nature, though a significant component in their structure is water (Wilkinson, 1958). The exopolysaccharides can be divided into two types, homopolysaccharides and heteropolysaccharides. Homopolysaccharides consist of polymers of a single sugar or amino-sugar residue. The glucans of various Streptococci species and Leuconostoc species are homopolysaccharides, as are the fructose polymers - levans - produced by both Gram-positive and Gram-negative organisms, e.g. Acetobacter species, Bacillus species and several plant pathogenic pseudomonads. Other specific bacteria have been reported to produce homopolysaccharides (Ward & Berkeley, 1980). Capsules and extracellular polymeric layers more often consist of heteropolysaccharides. Uronic acids and/or pyruval ketal groups are common constituents of heteropolysaccharides and confer a net negative charge to the polymer. Sutherland (1980) surveyed the constituents of a range of freshwater and marine bacterial exopolymers and found that the principal components were usually D-mannose, D-glucose, D-galactose and uronic acids. The degree of polymerization of heteropolysaccharides is lower than that of homopolysaccharides (Ward & Berkeley, 1980). Examples of heteropolysaccharides are the alginate polymers produced by Pseudomonads (Pringle et al. 1983a) and the xanthans of Xanthomonas species.

There is evidence to suggest that the capsule, or slime layer, does not completely shield the cell wall from the surrounding medium. The presence of capsules in several bacterial species does not prevent the formation of antibodies to cell wall components. Ward and Berkeley (1980) suggest that this is either due to an incomplete coverage by the capsular material so that the cell wall is exposed through 'holes', and/or due to cell wall components, e.g. phage-binding sites and surface antigen, penetrating the capsular material. Thus, even in the presence of exopolysaccharide layers the cell wall components may be capable of becoming involved in attachment interactions.

There are, then, a range of molecular types available at bacterial

cell surfaces for involvement in the interactions with and adhesion to a solid surface. There are polar and non-polar regions on amphiphatic molecules such as lipopolysaccharides and lipoproteins (Wood, 1980). These molecules are capable of a range of physico-chemical interactions, including polar and electrostatic interactions, and hydrogen bonding. The nature and dominance of the type of interactions is investigated in Chapter 5.

1.6 THE ROLE OF CELL SURFACE APPENDAGES IN BACTERIAL ATTACHMENT

The contribution made by bacterial cell surface structures in bacterial adhesion to solid surfaces has been investigated by several workers using electron microscopic techniques. Marine bacteria were found to have fibrils, pili, 'blebs' and 'droplets', and other appendages which were thought to affect their attachment (Corpe, et al. 1975; Paerl, 1975). Periphytic freshwater bacteria have also been shown to possess such appendages (Paerl, 1975)

It has more often been suggested that polymeric fibrils, pads or webs produced by bacteria are involved in bacterial adhesion. Paerl (1975) described slimy capsular secretions that were commonly produced by bacteria attached to particles in both marine and freshwater environments. A variety of marine periphytes, organisms such as pseudomonads, Caulobacter sp. Flavobacterium and Achromobacter sp, isolated from glass slides produced exopolymers (Corpe, 1973). Flexibacter aurantiacus CW7 and Hyphomicrobium vulgare ZV 580 were both found to produce 'adhesive' extracellular material (Marshall & Cruickshank, 1973). Organisms isolated from a polluted stream had no special attachment structures, but were enmeshed in extracellular material (Jones et al. 1969) and similar findings were indicated for attached bacteria from seawater samples (Marshall et al. 1971a). These exopolymeric materials have been proposed as important in the permanent irreversible attachment of bacteria to solid surfaces (Marshall et al. 1971a; Corpe, 1973; Fletcher & Floodgate, 1973). It had

further been suggested that exocellular polymeric materials may directly promote bacterial attachment to solid surfaces by acting as an adhesive (Corpe, 1974; Costerton et al. 1978). However, Paerl (1975) indicated that a number of attached bacteria had apparently no attachment appendages nor any adhesive polymeric material. He suggested that bacterial adsorption in these cases was molecular or chemical, occurring directly at the cell surface without the use of structural appendages. Similarly, a periphytic marine organism 'Pseudomonas atlantica' was able to produce large quantities of an extracellular polymeric material, mutants of this organism could not but were still able to attach to glass, indicating that polymeric material was not essential in the attachment of this bacterium (Corpe, 1980).

Thus the evidence supporting an adhesive function of exopolymers is primarily circumstantial, since it is partly based on the observations, described above, that periphytic bacteria are often enmeshed in a polymeric web. A feature not demonstrated by all attached bacteria. Recent evidence in fact indicates that some surface polymers may hold no such role and indeed may be inhibitory to bacterial attachment (Brown et al. 1977; Wardell et al. 1980 ; Pringle et al. 1983a).

However, certain bacterial surface structures may affect bacterial attachment to a solid/liquid interface and several different bacterial species possess apparently specialized attachment appendages. Structures such as flagella and fimbriae may be involved in adhesion to solid surfaces, facilitating attachment by overcoming the electrostatic repulsion barrier often existing between a bacterium and a solid substratum (Chapter 5).

Fimbriae, or pili, are long, straight and thin (10 nm) structures protruding from the cell wall surface of many different species, including members of the Enterobacteriaceae and Pseudomonads. The surface filaments are divided into several different classes depending on their characteristics. Type 1 fimbriae are adhesive allowing attachment to particles

and glass, etc. and they are pure proteins (Duguid, 1959). Other types of fimbriae vary in their adhesiveness or lack of adhesiveness. Many aquatic bacteria have been found to possess fimbriae (Corpe et al. 1975) which often cause them to form pellicles in broth at the liquid/air interface. The possession of fimbriae by aquatic bacteria may influence their attachment to solid surfaces.

Flagella have also been shown to be involved in bacterial attachment to substrata. Cells have been observed associated with a surface by the flagellum, sometimes with the body of the cell rotating round the axis of the adherent flagellum (Meadows, 1971; Sjoblad & Doetsch, 1983). The role of flagella in attachment may not only be as a component in the physico-chemical interaction between solid and bacterial surfaces, but also as an organelle of motility allowing chemotactic responses by bacteria. Both negative and positive chemotaxis is shown by motile bacteria (Adler et al. 1973; Doetsch and Seymour, 1970; Seymour & Doetsch 1973) and such chemotactic responses have been demonstrated as applying to solid substrata. Thus the macromolecular nutritive conditioning films may act to increase bacterial attachment by causing a positive chemotaxis on the part of the motile bacteria, while unfavourable compounds at surfaces may cause negative chemotaxis and less attachment (Young & Mitchell, 1973; Chet et al. 1975).

Some of the secondary oligotrophic bacterial colonizers of solid substrata possess specialized stalks and holdfasts which mediate their attachment. Caulobacter species have a stalk, which is continuous with the cell wall and at the end of which is holdfast material. It may be the secretion of this holdfast material which irreversibly attaches the Caulobacter to solid surfaces (Poindexter, 1981). Hyphomicrobium species apparently adhere via a polymeric material though they possess no holdfast structures (Marshall & Cruickshank, 1973). Another group

of bacteria with special attachment characteristics are the gliding bacteria. These undergo a temporary type of adhesion (Humphrey et al. 1979) and produce a polymeric slime which may aid gliding motility across a solid surface. Ridgway et al. (1975) described 'goblet'-shaped particles associated with the outer lipopolysaccharide component of the cell envelope of a marine gliding bacterium, Flexibacter polymorphus. These extended long filaments on fibrils which were proposed to be involved in attachment and gliding.

In this brief survey, the role of surface structures and appendages in bacterial attachment is evident. The specializations of some bacteria to surface 'life', for example that of the gliding bacteria which can move across a solid substratum, indicate a degree of adaptation to and selection for existence in interfacial environments. Such gliding bacteria may show different attachment characteristics from other copiotrophic bacteria, a possibility investigated in depth in Chapter 7.

Evidence for the significance of fimbriae and flagella in bacterial attachment in aquatic environments is scarce and structures such as holdfasts are limited to a few fairly specialized bacterial species. Further Marshall et al (1971a) suggested that the time periods involved in bacterial adsorption did not indicate involvement by pili or holdfast structures. Once more, then, one can return to the consideration of the bacterial cell surface as the major determinant in attachment.

1.7 THE INFLUENCE OF SOLID/LIQUID INTERFACES ON BACTERIAL MORPHOLOGY, PHYSIOLOGY AND SURVIVAL

Solid/liquid interfaces represent unique habitats for bacterial invasion and colonization. They possess features not encountered by the bacteria in the bulk liquid phase. Not only do environmental factors such as pH and electrolyte concentration differ but the solid surface itself will have a variety of effects on the attached bacteria. The effects on the bacteria may be both morphological and/or metabolic, and may vary from species to species.

The accumulation of large amounts of exocellular polymeric material,

often associated with attached bacteria, represents a major morphological change by the bacteria. Such extensive polymeric webs are usually not found in the bulk aqueous phase, though flocculation of bacteria is often related to the presence of polymer (Harris & Mitchell, 1973). Filamentous bacterial forms become apparent on solid surfaces at approximately the same time as secondary colonization occurs (Characklis, 1980). These may either represent invasion of the substratum by new bacterial species or may be the result of changes in the morphology of previously attached bacteria. Similarly the often very small copiotrophic bacteria which represent the primary colonizers are displaced by normal size bacteria after approximately 12 hours (Marshall et al. 1971b). Some copiotrophic bacteria divide to form small cells under oligotrophic conditions (Kjelleberg et al. 1982). Marshall (1979) suggested that the small primary colonizers are starved cells and that their apparent replacement by larger cells may be the result of growth at the interface. Kjelleberg et al. (1982) showed that small starved cells grew on the nutrients accumulated at solid surfaces.

Evidence for the affect of attachment on bacteria changing with the bacterial type was indicated by Humphrey et al. (1983). The 'dwarfing' of cells under starvation conditions varied between the liquid phase and the solid/liquid interface and with the bacterial type. Twelve hydrophilic bacilli decreased in size more rapidly at a solid surface than in the liquid phase while the reverse was true of three hydrophobic rod-shaped bacteria. Three coccoidal bacteria did not decrease in size either at the surface or in the aqueous medium. The authors suggested that these differences may have been due to different abilities on the part of the organisms to utilize nutrients in the conditioning film, or due to different effects of solid and liquid surface tensions.

Much of the early investigation into the relationship between solid surfaces and bacteria concentrated on the effects of surfaces on bacterial activity and growth. Heukelekian and Heller (1940) showed that at low

peptone concentrations (< 0.5 mg/l) E. coli could not grow in the absence of glass beads. It was found that this 'surface effect' varied with both the concentration and type of organic matter present, and the solid surface involved (ZoBell, 1943). For example, concentrations of $> 5-10$ mg/l caused no surface effect, while some organics, e.g. lignoprotein increased bacterial activity while others did not, e.g. glucose. ZoBell (1943) suggested that the surface effect might be due to the accumulation of nutrient molecules at the solid surfaces and/or that the solid surfaces stopped the removal of exoenzymes and hydrolysates from the periphytes and possibly influenced the orientation of enzymes.

In natural aquatic systems attached bacteria have been found, generally, to be more active than those free-living in the bulk phase (Hendricks, 1974; Harvey & Young, 1980). However the situation may vary with the nature of the solid surface and the organic molecules forming the conditioning film. Fletcher (1979b) found no general 'surface effect' for a range of substrata for the uptake of amino acids by attached marine bacteria.

Surfaces may not only influence nutrient availability, but factors such as pH and electrolyte concentration will differ from the bulk phase. These might either promote or inhibit bacterial growth and may provide different micro-niches for a variety of bacterial specialists. Surfaces may not only accumulate nutrients for, but also inhibitors to, growth. This becomes especially likely as the bacterial film develops and the community becomes more diverse, since waste products of microbial metabolism will become immobilized in the vicinity of bacteria. Bacterial attachment interactions will also influence the bacterial cell membrane by changing both membrane structure and charge density. This may well affect bacterial physiology, since the bacterial envelope represents the 'organelle' of substrate and electron transport.

In conclusion, solid surfaces exert a considerable influence on attached bacteria. The organic conditioning film present at the surface is a potential nutrient source for bacteria, though its degradation appears to vary with the nature of the solid substratum, the type of organic molecules and the bacterial species involved in the interaction. The presence of the organic conditioning film may confer a further advantage on bacterial immobilization on surfaces since the search for food may be unnecessary and there would be a net conservation of energy (Fletcher & Marshall, 1983). It has also been suggested that solid surfaces may present protection from phage attack (Roper & Marshall, 1974) and in fast flowing aquatic habitats bacterial attachment may offer a selective advantage since it avoids bacterial wash-out (Costerton et al. 1978; Ellwood et al. 1982).

There are, however, potential disadvantages to biofilm formation. As the film develops and surface nutrients become depleted so diffusion of nutrients and oxygen from the bulk phase becomes essential, but diffusion itself becomes limited by film thickness. Bacterial competition for nutrients etc. will become more intense and the possibly inhibitory end-products of bacterial metabolism more plentiful.

The initiation and development of the association between solid surfaces and bacteria, then, are complex. The interactions can be roughly divided into two phases, the first being the events of bacterial attachment, the second the growth and development of a biofilm. The two stages in biofilm development are not separate; the first will overlap the second (Characklis, 1980). An understanding of the initial attachment interactions may lead to a capability for controlling detrimental fouling in man-made structures and a better grasp of the environmental influences on bacterial adhesion in natural aquatic systems.

It is this first phase, the mechanisms of bacterial attachment, which is investigated in this thesis.

CHAPTER TWO

CHARACTERIZATION AND ATTACHMENT OF SELECTED FRESHWATER BACTERIA

2.1 AIMS

To identify a selection of bacteria isolated from a freshwater stream and to determine their levels of attachment to a hydrophobic and to a relatively hydrophilic polystyrene surface.

2.2 INTRODUCTION

A range of bacteria were isolated from freshwater and were identified using a range of biochemical tests. The attachment of the isolated species to a hydrophobic and a relatively hydrophilic polystyrene surface was investigated to determine differences in bacterial attachment with solid substratum. The extent of the differences in attachment between species was investigated as well as whether there was a relationship in attachment levels within species.

2.3 MATERIALS AND METHODS2.3.1 Organisms

A selection of bacteria were isolated from fast-running freshwater at Baginton Weir, River Sowe (Pringle & Fletcher, 1983b) after attaching to solid surfaces suspended beneath the water. Thirteen of these isolates were selected for identification and biochemical characterization.

2.3.2 Medium and Storage Conditions

The bacteria to be characterized were grown in a broth medium (PYE) containing 0.1% (w/v) peptone (Oxoid Ltd., London) and 0.07% (w/v) yeast extract powder (Lab M, London) in distilled water, adjusted to pH 7.4 with 1M NaOH (BDH Chemicals Ltd, Poole). Stock cultures, after growth at 15°C for 16 hrs on a rotary shaker at 150 rpm, were maintained at 4°C for 6 weeks. Agar plates and slopes consisted of this medium and 0.75% (w/v) agar (No.2 Lab M, London). Slopes of bacterial cultures were kept at 4°C and sub-cultured every 3 months.

2.3.3 Identification and Biochemical Characterization Methods

(1) Gram stain and cell morphology.

Bacteria grown on PYE agar plates (Section 2.3.2) were Gram stained after heat-fixing air-dried bacteria on a glass slide. They were then investigated for their Gram stain reaction and cell morphology under a light microscope (Kyowa optical) at a magnification of X1000.

(2) Bacterial Motility.

Bacteria were grown in broth culture (Section 2.3.2) at 15°C on a rotary shaker (150 rpm) for 14 hrs. A drop of culture was suspended from a coverslip in the form of a hanging drop. This was observed microscopically (Kyowa optical light microscope) at a magnification of X400 to determine the motility of the bacterium in the aqueous phase.

The ability of a bacterium to glide across a solid surface was investigated by growing the organism on a low nutrient agar medium (PYE) (the basic medium was used; Section 2.3.2) at 15°C for 72 hrs. Bacterial colonies were observed for spreading, and the nature of the spreading was determined using a bifocal microscope (Olympus, Japan).

(3) Growth Form in Broth Cultures.

An inoculum of 0.1 ml of bacterial stock culture was placed in fresh PYE medium (Section 2.3.2). The culture was then grown at 15°C in an orbital incubator rotating at 150 rpm for 24 hrs. The presence or absence of aggregation was noted over the whole growth period.

(4) Pigment Production.

(a) bacteria were grown on the PYE agar medium (Section 2.3.2) at 15°C for 72 hrs and observed for pigment production.

(b) possible Pseudomonas species were investigated further for diffusible pigment production. The media developed by King et al (1954) were used to determine the production of pyocyanin and fluorescein. The medium for enhanced pyocyanin production, Kings A medium, consisted of protease peptone (20g) (Difco Lab, Michigan); glycerol (10g) (Fisons Scientific Apparatus, Loughborough); K₂SO₄, anhydrous (10g) (BDH

Chemicals Ltd., Poole); MgCl_2 anhydrous (1.4g) (May & Baker Ltd., London); agar (20g), (No.2, Lab M., London) in 1 litre of distilled water adjusted to pH 7.2. The medium was sterilized by autoclaving (121°C , 10 mins). Agar plates of this medium were streak inoculated with bacteria and incubated at 15°C for 72 hrs. The cells were harvested with 10 mls chloroform (J. Burrough Ltd., London) and slightly acidified by the addition of 1M HCl (May & Baker Ltd., London). The production of a red colour indicated the presence of pyocyanin typical of Pseudomonas aeruginosa.

The second medium, Kings B medium, demonstrated the production of fluorescin. It consisted of protease peptone (20g) (Oxoid Ltd., London); glycerol (10g) (Fisons Scientific Reagents, Loughborough); K_2HPO_4 (1.5g) (Fisons Scientific Reagents, Loughborough); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.5g) (Fisons Scientific Reagents, Loughborough); agar (20g) (No.2, Lab M., London) dissolved in 1 litre of distilled water adjusted to pH 7.2. The medium was autoclaved at 121°C for 10 mins. After streak inoculating the plates and incubating at 15°C for 72 hrs, the colonies were observed under ultra violet illumination ($\lambda = 365 \text{ nm}$) for fluorescence. A positive reaction indicated the fluorescin production of Pseudomonas species.

(5) Oxidase Test.

Pure cultures of bacteria were inoculated onto PYE agar plates (Section 2.3.2) and incubated at 15°C for 72 hrs. A colony was picked from the agar with a glass rod and spotted onto filter paper impregnated with a 1% solution tetramethyl-p-phenylene diamine hydrochloride (Sigma Chemical Company, Poole) in distilled water. A positive violet colour within 5-10 secs indicated cytochrome oxidase activity (Kovacs, 1956).

(6) Catalase Test.

Colonies of pure bacterial cultures grown on PYE agar medium (Section 2.3.2) at 15°C for 72 hrs were spotted, with a sterile wooden stick, onto a 30% hydrogen peroxide solution (Fisons Scientific Reagents, Loughborough)

in distilled water on a glass slide. A positive reaction, showing the presence of catalase, was demonstrated by the immediate production of bubbles (MacFaddin, 1976).

(7) Sensitivity to Vibriostatic Agent O/129.

Suspected species of *Vibrios* were tested for their sensitivity to a phosphate derivative of 2,4-diamino-6,7-diisopropyl-pteridine (BDH Chemicals Ltd., Poole), i.e. the vibriostatic agent O/129 (Shewan et al. 1954). Filter paper discs 6 mm in diameter were sterilized by autoclaving (121°C, 10 mins). Filter sterilized O/129 (0.45 µ cellulose acetate filter, Millipore) was then added aseptically to the discs so that each contained a concentration of 150 µg. A lawn of a pure bacterial culture was grown on agar plates consisting of 0.05% (w/v) peptone, 0.035% (w/v) yeast extract powder, 0.75% (w/v) agar (No.2, Lab M) in distilled water, adjusted to a pH of 7.4 by 1M NaOH. The vibriostatic agent was placed on the plates before growth was initiated. After 48 hrs at 15°C, sensitivity or resistance to O/129 was determined (Furniss et al. 1978). *Vibrios* are considered to be sensitive to this agent.

(8) Growth on a Single Carbon Source.

Using a basal medium devised by Palleroni and Doudoroff (1972) the bacterial species to be characterized were investigated for their ability to grow on a range of carbon substrates. The basal medium consisted of phosphate buffer (M/30) adjusted to pH 6.8; 0.1% (w/v) NH_4Cl (Fisons Scientific Reagents, Loughborough); 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough); 0.005% (w/v) ferric ammonium citrate (BDH Chemicals Ltd., Poole); 0.0005% (w/v) CaCl_2 (Fisons Scientific Reagents, Loughborough); and 1% (w/v) Ionagar (No.2, Oxoid Ltd., London). The ionagar in phosphate buffer was autoclaved (121°C, 15 mins) allowed to cool and a filter sterilized (0.45 µ cellulose acetate filter) mixture of the salt solution was added. Individual members of a selection of filter sterilized (0.45 µ cellulose acetate filter) carbon sources, at a concentration of 0.1% (w/v), were also added to the agar basal medium

and poured as plates. The carbon sources used were glucose (Fisons Scientific Reagents, Loughborough); mannitol (Fisons Scientific Reagents, Loughborough); maltose (Fisons Scientific Reagents, Loughborough); ethanol (J. Burrough Ltd., London); cellobiose (BDH Chemicals Ltd., Poole); galactose (BDH Chemicals Ltd., Poole); xylose (Sigma Chemical Company, Poole); arabinose (BDH Chemicals Ltd., Poole); lactose (Fisons Scientific Reagents, Loughborough) and inositol (Sigma Chemical Company, Poole).

The single carbon source agar plates were streak-inoculated with pure cultures of the bacteria and growth was monitored over a 2 week period at 15°C. The extent of growth was compared with a negative control, a plate of basal medium containing no carbon source.

(9) Polymer hydrolases.

The ability of the organisms to hydrolyse a series of substrates, i.e. gelatin, starch, casein, cellulose, and deoxyribose nucleic acid was investigated (Blazovic & Grace, 1975; McFaddin, 1976).

(a) Gelatin hydrolysis was demonstrated by growing pure cultures on the PYE medium agar (Section 2.3.2) supplemented with 1% (w/v) gelatin (Fisons Scientific Reagents, Loughborough). Plates were incubated for 5 days at 15°C and then flooded with mercuric chloride solution (12g mercuric chloride (Fisons Scientific Reagents, Loughborough), 80 ml distilled water, 16 ml HCl (BDH Chemicals Ltd., Poole)). A clear zone developing around the colonies indicated a positive reaction, i.e. the ability to proteolyse gelatin.

(b) The hydrolysis of starch was demonstrated by the addition of a 0.8% (w/v) soluble starch (BDH Chemicals, Poole) to the PYE agar medium (Section 2.3.2). After streak inoculating agar plates and incubating at 15°C for 5 days, starch hydrolysis was determined by flooding the plates with Gram's iodine (10g iodine, (Fisons Scientific Reagents, Loughborough); 20g potassium iodide (Fisons Scientific Reagents, Loughborough) in 1 litre

distilled H_2O). A positive reaction, i.e. starch hydrolysis, was shown by a colourless zone forming around bacterial colonies in a blue stained medium.

(c) A similar procedure was developed to investigate casein and cellulose hydrolysis. The PYE medium agar (Section 2.3.2) contained 10% (w/v) dried skimmed milk, sterilized separately and mixed on pouring, or 10% (w/v) cellulose (Sigma Chemical Company, Poole) respectively. The plates were streak inoculated and grown at $15^{\circ}C$ for 5 days. A clear zone round the colonies indicated a positive reaction, i.e. either casein or cellulose hydrolysis.

(d) DNAase activity was investigated by using DNA agar (Oxoid Ltd, London). Plates of pure streak cultures were incubated for 5 days at $15^{\circ}C$ and then flooded with 1N HCl. DNA hydrolysis was shown by a clear zone around the colonies.

(10) Oxidative and Fermentative Metabolism of Carbohydrates.

Oxidation-fermentation tests were performed using the Hugh Leifson method (Hugh & Leifson, 1953). A basal medium was prepared containing 2.0g peptone; 5.0g NaCl (Fisons Scientific Reagents, Loughborough); 0.3g K_2PO_4 (Fisons Scientific Reagents, Loughborough); 2.0g agar (No.2, Lab M) and 0.0025% (w/v) bromocresol purple (Maynard & Baker Ltd., London) in 1000 ml of distilled water adjusted to pH 7.1. After autoclaving ($121^{\circ}C$, 10 mins) and cooling, a 1% (w/v) solution of filter sterilized (0.45 μ cellulose acetate filter) glucose solution was added. The medium was poured aseptically into sterile test tubes, half of which were then sealed with sterile paraffin oil. Two tubes, one unsealed and one sealed, were stab inoculated with pure cultures of each organism. Oxidative or fermentative metabolism was then determined by the reactions in both tubes after up to 5 days incubation at $15^{\circ}C$. Production of a yellow colour in the open tube indicated oxidation of glucose, in the sealed tube fermentation of glucose.

(11) Urease Activity.

The hydrolysis of urea was determined by using a medium consisting of a phosphate buffer adjusted to pH 6.8, 0.1g yeast extract; 20g urea (BDH Chemicals Ltd., Poole); and 0.2g (w/v) phenol red (Sigma Company, Poole) in 1000 ml distilled water. The medium was filter sterilized (0.45 μ cellulose acetate filter) and pipetted aseptically into sterile 5 ml screw-top bottles. The broths were inoculated and incubated at 15°C for up to 72 hrs. The production of a red colour indicated a positive result, i.e. urease activity (Blazevic & Grace, 1975; MacFaddin, 1976).

(12) β -galactosidase Activity.

β -galactosidase activity for each isolate was investigated using a basal medium (MacFaddin, 1976) of peptone water which contained 10g peptone; 5g NaCl in 1000 ml of distilled water adjusted to pH 7.4. A filter sterilized solution (0.45 μ cellulose acetate filter) of 6.0g O-nitrophenyl- β -D-Galactopyranoside (Sigma Chemical Company, Poole) in 1 litre of 0.01M phosphate buffer adjusted to pH 7.5 was added aseptically in 250 ml portions to 750 ml of peptone water. Quantities (2.5 ml) of this broth were then aseptically placed in sterile tubes. The broths were inoculated for up to 72 hrs at 15°C. β -galactosidase activity was indicated by the formation of a yellow colour.

(13) Phosphatase Activity.

Acid, neutral and alkaline phosphatase activity was investigated by using a substrate of 1% (w/v) 4-nitrophenyl disodium orthophosphate (PNPP) (Fisons Scientific Reagents, Loughborough) in distilled water. The tests were carried out in a reaction mixture of 0.3 mls 1% PNPP and 2.7 mls of 0.175M Tes, Tris, Acetic acid buffer (TTA) (1M TTA consists of 7.64g Tes acid (Sigma Chemical Company, Poole); 4.06g Tris base (Sigma Chemical Company, Poole); 2.1 ml Glacial acetic acid (Fisons Scientific Reagents, Loughborough); in 100 ml distilled water). Acid phosphatase activity was determined by adjusting the pH of the TTA buffer to 5 and adding sodium orthovanadate (BDH Chemicals Ltd., Poole) to a final concentration of 10^{-5} M and pre-incubating an inoculum of cells with

ethylenediaminetetraacetic acid, EDTA (BDH Chemicals Ltd., Poole) to a concentration of 10^{-5} M for 20 mins. The TTA buffer was adjusted to a pH of 7 for neutral phosphatase activity with the addition of sodium fluoride (Fisons Scientific Reagents, Loughborough) to a concentration of 10^{-3} M and pre-incubating the inoculum with EDTA to a final concentration of 10^{-5} M for 20 mins. The presence of alkaline phosphatases was shown by buffering the reaction mixture with TTA at pH 9 and adding sodium orthovanadate to a final concentration of 10^{-5} M and sodium fluoride to a concentration of 10^{-3} M. Sodium orthovanadate blocks the activity of neutral phosphatases while sodium fluoride and EDTA block acid and alkaline phosphatases respectively.

The three reaction mixtures were equilibrated at 30°C in a water bath for 30 mins before adding a test sample. One ml of inoculum from a PYE medium broth culture (Section 2.3.2) grown for 16 hrs at 15°C in a rotary shaker at 150 rpm was added to each of the three reaction mixtures. The tests were incubated in a water bath at 30°C for up to 24 hrs with the addition of 0.1 ml toluene (May & Baker Ltd., London) to prevent cell growth. Phosphatase activity was indicated by the formation of a yellow colour.

(14) Dihydrolase and Decarboxylase Activity.

Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities of the bacterial isolates were investigated by using a modification of Møllers decarboxylase base (MacFaddin, 1976). The base contained 0.5g peptone; 5g beef extract (Difco Labs, Detroit); 0.005g cresol red (Sigma Chemical Company, Poole); 5.0mg pyridoxal (Sigma Chemical Company, Poole); 0.1g bromocresol purple and 0.5g glucose in 1000 ml distilled water adjusted to pH 6.0. To this base solution was added either 1% (w/v) L(+) lysine dihydrochloride (Sigma Chemical Company, Poole); 1% (w/v) L(+) arginine monohydrochloride (Sigma Chemical Company, Poole); or 1% (w/v) L(+) ornithine dihydrochloride (Sigma Chemical

Company, Poole). The media were then filter sterilized (0.45µ cellulose acetate filter) and 3 ml portions were aseptically put in 5 ml sterile tubes which were then lightly inoculated and overlaid by 1 ml of sterile paraffin. The tests were incubated at 15°C for up to 10 days. A positive reaction, that is dihydrolase or decarboxylase activity, was shown by a turbid purple or yellow-purple colour.

(15) Indole Reaction.

The indole reaction (MacFaddin, 1976) was used to investigate the isolates' ability to break down tryptophan. The test solution consisted of 1g peptone; 5g NaCl (Fisons Scientific Reagents, Loughborough); in 1000 ml distilled water adjusted to pH 7.4, supplemented by 1% (w/v) tryptophan (Sigma Chemical Company, Poole). This was put into tubes in 3 ml portions and sterilized by autoclaving (121°C, 15 mins). The broths were inoculated and incubated at 15°C until the culture had grown to an optical density in a colorimeter > 0.1 at 540 nm (Corning Colorimeter 252). Tryptophanase activity was then indicated by the formation of a red colour on the addition of 0.5 ml Kovacs Reagent. Kovacs Reagent contained 10g p-dimethylaminobenzaldehyde (Sigma Chemical Company, Poole) dissolved in 150 ml butyl alcohol (Fisons Scientific Reagents, Loughborough) in a water bath at 56°C with the addition of 50 ml conc HCl (BDH Chemicals Ltd., Poole).

(16) Analytical Profile Index (API)

The API 20E system (API Laboratory Products Ltd., Basingstoke) consists of a series of conventional biochemical tests commonly used to identify Enterobacteriaceae and other Gram-negative bacteria. The tests are in a miniaturized version and were inoculated from a bacterial colony grown on PYE agar medium (Section 2.3.2) which had been suspended in 10 mls of sterile distilled water. The test strips were incubated at 15°C for up to 72 hrs before the biochemical test reagents were added. A purity plate (PYE) for each test suspension was also inoculated

and incubated. The biochemical tests included β -galactosidase activity; dehydrolase and decarboxylase activity; a citrate test; hydrogen sulphide production; urease activity; tryptophane deaminase test (TDA); indole reaction; Voyes-Proskaver reaction (VP); gelatin liquifaction; the utilization of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and L(+) arabinose; oxidase reaction; and catalase reaction.

Of these biochemical tests, those which have not been described in Sections 2.33 (1)-(15) require a brief explanation. The citrate test determined if the bacterial species could utilize citrate, in the form of sodium citrate, as the sole carbon source. The production of hydrogen sulphide from thiosulphate showed the organism was able to metabolise this substrate. Tryptophane deaminase activity was indicated by a positive TDA test after the addition of 1 drop of 10% ferric chloride (API Laboratory Products Ltd., Basingstoke) to the test ampule. A brown red colour indicated the production of indole pyruvic acid and the breakdown of tryptophane. A positive pink VP reaction produced by the addition of 1 drop of 40% potassium hydroxide (API Laboratory Products Ltd., Basingstoke) followed by 1 drop of 6% alpha-naphthol (API Laboratory Products Ltd., Basingstoke) to a test ampule containing sodium pyruvate, showed the presence of acetoin. This is an intermediate of glucose metabolism. The utilization, oxidation or fermentation of single carbon sources included additional substrates to those already described, i.e. rhamnose, sucrose, melibiose and amygdalin.

2.3.4 Growth rates

The specific growth rates of individual species were estimated for bacteria grown in the PYE medium broth (Section 2.3.2) and a glucose minimal medium. The glucose medium contained 0.544g KH_2PO_4 (Fisons Scientific Reagents, Loughborough); 0.2g glucose (Fisons Scientific Reagents, Loughborough); 0.38g NH_4Cl (Fisons Scientific

Reagents, Loughborough); and 0.6 ml of a salt solution consisting of $10 \text{ g l}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough); $1.0 \text{ g l}^{-1} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$ (May & Baker Ltd., London); $0.4 \text{ g l}^{-1} \text{ FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough) and $0.1 \text{ g l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough) in 100 ml distilled water adjusted to pH 7.4 by 1M NaOH. The glucose and NH_4Cl were autoclaved separately (121°C , 10 mins) and added aseptically to the buffer (KH_2PO_4) which had been sterilized by autoclaving (121°C , 15 mins). Filter sterilized salt solution (0.45 μ porosity cellulose acetate filter) was then added so the total medium volume was 100 mls.

The growth rate of the bacteria was determined by inoculating 1 ml of stock culture (Section 2.3.2) into 100 mls of PYE medium broth and incubating at 15°C in an orbital incubator at 150 rpm. Growth was followed turbidometrically at 540 nm with a Corning colorimeter 252. To determine growth rates in glucose minimal medium the bacteria were initially grown for 16 hrs in this medium and then re-inoculated in 1 ml aliquots into fresh minimal medium. The procedure was then identical to that for PYE medium broth.

2.3.5 Attachment Assay

Pure cultures of bacteria were grown to early stationary phase in PYE medium broth (Section 2.3.2) at 15°C in an orbital incubator rotating at 150 rpm. The bacteria were then harvested by centrifugation, 10,960 av.g for 15 mins (Beckman, Model J-21B centrifuge) and resuspended in 0.01M HEPES buffer (Sigma Chemical Company, Poole), adjusted to pH 7.4 with 1M NaOH. The cells were washed once by centrifugation to remove any remaining medium and then resuspended in 0.01M HEPES buffer, pH 7.4 and PYE medium broth (Section 2.3.2), pH 7.4. Cell numbers were controlled by measuring the absorbance of the buffer and medium at 540nm in a Corning colorimeter 252. Unless otherwise stated in the results, for all suspensions, A_{540} was 0.1.

5 ml aliquots of the suspensions were then transferred to 5 cm polystyrene (PD) petri dish (Sterilin, Teddington, England), a hydrophobic surface, or tissue culture treated polystyrene (TCD) (Costar, Cambridge, Mass.), a relatively hydrophilic surface; these served as the attachment substrata. Duplicate PD and TCD petri dishes were then incubated for 1 hr at 15°C, at the end of which time they were washed 3 times with 0.01M HEPES buffer (pH 7.4) to remove loosely attached bacteria. The remaining attached bacteria were then fixed with Bouins fixative (71% v/v saturated aqueous picric acid (Harris Biological Supplies Ltd., Weston-Super-Mare); 24% v/v formalin (Fisons Scientific Reagents, Loughborough); 5% v/v acetic acid (Fisons Scientific Reagents, Loughborough) and stained with crystal violet (5g/l) (Fisons Scientific Reagents, Loughborough) (Fletcher, 1976; 1977). Bacterial attachment was estimated indirectly by measuring absorbance at 590 nm on a spectrophotometer (Unicam Instruments, Cambridge), of the stained attached bacteria (590 nm is the adsorption maximum of crystal violet). Four readings of randomly selected areas were taken from each of the duplicate dishes.

2.4 RESULTS

The selected species were identified as four Acinetobacter spp., two Aeromonas hydrophila, a Chromobacterium sp, a coryneform, and Enterobacter cloacae, Pseudomonas aeruginosa, Pseudomonas fluorescens and a Flexibacter sp. (Appendix Tables 1 to 4 show the identification results). (Skinner & Lovelock, 1979; MacFaddin, 1976; Blazovic & Grace, 1975).

The majority of the isolates were asporogenous, Gram-negative bacilli, the only exception being a Gram-positive coryneform. This predominance of Gram-negative bacteria may reflect the species composition of the total attached bacterial isolates from the River Sowe, i.e. Pseudomonas spp, 30%; Aeromonas spp, 26%; yellow rods, 12%; Acinetobacter spp, 4%; Flexibacter spp, 4%; Chromobacterium spp, 4%; and Bacillus

spp, 2% (Pringle & Fletcher, 1983b). The attachment assays (Table 2.1 and Table 2.2) show a wide divergence between different bacterial species in attachment levels to the two polystyrene surfaces. Low attachment levels are indicated by $A_{590} \times 10^3 < 50$, moderate levels by levels between 50 and 150 and high levels by absorbance > 150 .

There was no relationship between generic classification of the isolates and the extent of their attachment, which varied considerably within species (Table 2.1), e.g. Acinetobacter calcoaceticus (H_3 , H_{42}), Aeromonas hydrophila (H_{22} , H_{48}). There was also no consistency in interaction with the two polystyrene surfaces, that is the hydrophobic PD surface the relatively hydrophilic TCD surface, the same species showing widely different levels in attachment to each surface, e.g. Acinetobacter calcoaceticus (H_3 , H_{42}) and Aeromonas hydrophila (H_{22} , H_{48}).

Both motile and non-motile bacteria were capable of high and low levels of attachment, e.g. the motile Aeromonas hydrophila (H_{48} and H_{22}), and the non-motile Acinetobacter calcoaceticus (H_3) and Acinetobacter sp. (H_7) (Table 2.1). There was also no apparent relationship between the metabolic capabilities, i.e. the number of substrates a bacterium could utilize, and bacterial attachment to the solid surfaces (Table 2.1 and 2.2, Appendix Tables 1-4).

The bacteria demonstrated a range of attachment levels to the hydrophobic polystyrene surface (PD) and the relatively hydrophilic surface (TCD). In the buffered suspensions, Acinetobacter calcoaceticus (H_3) showed the same level of attachment to both surfaces, while Chromobacterium sp (H_{31}) attached in greater numbers to the PD surface. Acinetobacter calcoaceticus (H_{42}) showed far better attachment to the hydrophilic TCD surface than the hydrophobic PD surface. The presence of nutrients while the bacteria were attaching also had a considerable impact on their attachment levels. Some species attached in greater numbers in nutrient than buffer, e.g. Chromobacterium sp (H_{31}), while

TABLE 2.1 The Attachment of Selected Bacteria to Polystyrene Surfaces in a Buffer^a suspending medium (2 separate experiments)

Organism Number	Species	Attachment ($A_{590} \times 10^3$ of Attached Bacteria)				
		PD ¹	113 (\pm 13)	185 (\pm 25)	125 (\pm 7)	TCD ²
H3	Acinetobacter calcoaceticus	162 (\pm 17) ^b				
H7	Acinetobacter sp	16 (\pm 3)	14 (\pm 5)	24 (\pm 3)	16 (\pm 3)	
H20	Acinetobacter sp	23 (\pm 11)	104 (\pm 3)	84 (\pm 13)	48 (\pm 14)	
H34	Acinetobacter sp	9 (\pm 3)	18 (\pm 3)	16 (\pm 3)	42 (\pm 4)	
H42	Acinetobacter calcoaceticus	14 (\pm 3)	21 (\pm 8)	73 (\pm 8)	74 (\pm 5)	
H22	Aeromonas hydrophila	16 (\pm 3)	21 (\pm 3)	21 (\pm 3)	26 (\pm 3)	
H48	Aeromonas hydrophila	136 (\pm 19)	188 (\pm 10)	130 (\pm 7)	166 (\pm 5)	
H31	Chromobacterium sp	79 (\pm 13)	65 (\pm 10)	12 (\pm 3)	19 (\pm 4)	
H16	Coryneform sp	10 (\pm 4)	12 (\pm 3)	18 (\pm 3)	18 (\pm 3)	
H12	Enterobacter cloacae	126 (\pm 15)	144 (\pm 10)	27 (\pm 3)	69 (\pm 10)	
H15	Pseudomonas aeruginosa	137 (\pm 21)	91 (\pm 10)	80 (\pm 10)	63 (\pm 13)	
H2	Pseudomonas fluorescens	43 (\pm 2)	220 (\pm 40)	41 (\pm 4)	26 (\pm 5)	
H38	Flexibacter sp	319 (\pm 14)	309 (\pm 20)	275 (\pm 18)	336 (\pm 13)	

^a0.01M HEPES pH 7.4

^bParenthetical values represent the 95% confidence limits of the mean (n = 8)

¹PD Polystyrene petri dish

²TCD Tissue Culture petri dish

TABLE 2.2 The Attachment of Selected Bacteria to Polystyrene Surfaces in a Nutrient^a suspending medium (2 separate experiments)

Organism Number	Species	Attachment ($A_{590} \times 10^3$ of Attached Bacteria)				
		PD ¹	PD ¹	TCD ²	TCD ²	TCD ²
H ₃	Acinetobacter calcoaceticus	128 (\pm 20) ^b	186 (\pm 26)	198 (\pm 60)	371 (\pm 57)	
H7	Acinetobacter sp	7 (\pm 3)	14 (\pm 4)	14 (\pm 5)	25 (\pm 3)	
H20	Acinetobacter sp	10 (\pm 5)	106 (\pm 20)	29 (\pm 9)	40 (\pm 13)	
H34	Acinetobacter sp	12 (\pm 3)	28 (\pm 5)	21 (\pm 3)	53 (\pm 5)	
H42	Acinetobacter calcoaceticus	9 (\pm 4)	40 (\pm 17)	83 (\pm 4)	96 (\pm 4)	
H22	Aeromonas hydrophila	30 (\pm 7)	29 (\pm 5)	78 (\pm 20)	63 (\pm 8)	
H48	Aeromonas hydrophila	92 (\pm 16)	134 (\pm 20)	118 (\pm 11)	157 (\pm 16)	
H31	Chromobacterium sp	282 (\pm 30)	309 (\pm 38)	98 (\pm 38)	122 (\pm 32)	
H16	Coryneform sp	14 (\pm 3)	10 (\pm 3)	35 (\pm 14)	6 (\pm 5)	
H12	Enterobacter cloacae	97 (\pm 14)	117 (\pm 20)	23 (\pm 8)	24 (\pm 3)	
H15	Pseudomonas aeruginosa	138 (\pm 37)	106 (\pm 22)	167 (\pm 25)	274 (\pm 53)	
H2	Pseudomonas fluorescens	162 (\pm 26)	208 (\pm 30)	235 (\pm 20)	260 (\pm 25)	
H38	Flexibacter sp	33 (\pm 3)	208 (\pm 14)	42 (\pm 4)	174 (\pm 15)	

^a0.1% (w/v) peptone, 0.07% (w/v) yeast extract, pH 7.4

^bParenthetical values represent the 95% confidence limits of the mean (n = 8)

¹PD Polystyrene petri dish

²TCD Tissue Culture petri dish

in other species attachment was inhibited by the presence of nutrients e.g. the Flexibacter sp. (H₃₈). In yet other interactions, attachment was either increased or decreased to one surface while that to the other substratum was largely unchanged, e.g. Pseudomonas aeruginosa (H₁₅) (Table 2.1 and 2.2). The attachment levels of a few bacteria were not significantly different (95% confidence limits of the mean) in either buffer or nutrient solution, e.g. Acinetobacter sp. H₃₄).

A comparison of the duplicate attachment experiments (Table 2.1 and 2.2) shows that the attachment levels of individual test organisms to PD and TCD were different between experiments, e.g. Acinetobacter sp (H₂₀) showed relatively low levels of attachment to the PD surface and high levels to the TCD surface in the first experiment (Table 2.1), while in the second experiment (Table 2.1), this bacterium showed the reverse relationship. Although the inoculation procedure and growth conditions were identical for each experiment, the final optical density of the cultures was not determined. The bacteria may, then, have been at a slightly different growth stage, which could influence the levels of attachment.

2.5 DISCUSSION

The initial colonizers of solid surfaces, in both marine and freshwater environments, have often been found to consist of Gram-negative asporogenous rods with Pseudomonas sp, Aeromonas and pigmented forms, e.g. Flavobacteria sp. and Achromobacter sp. among the most common species (Batoosingh & Anthony, 1971; Corpe, 1974a). This is true for a range of solid surfaces from polytetrafluoroethylene (PTFE) and polystyrene (Pringle & Fletcher, 1983b) (the isolates used in this work) to glass microscope slides (Corpe, 1974a, Pringle & Fletcher, 1983b) and pebbles (Batoosingh & Anthony, 1971). The apparent predominance of Gram-negative bacteria on solid surfaces may partly reflect their numerical superiority in the liquid phase. There are, however, considerable difficulties in bacterial isolation procedures from natural

environments. Since some bacteria can be extremely fastidious in their nutrient requirements the media selected for isolation may not be appropriate for their growth and survival; thus, these organisms will not be represented in any estimate of the bacterial population.

The wide range of bacterial attachment levels demonstrated in this Chapter have also been found by other workers using a variety of bacterial species. Zvyagintsev (1959) showed a selection of bacteria capable of a range of interactions with glass surfaces from weak to strong. This work also found no relationship between generic classification and attachment, and no difference between Gram-positive and Gram-negative bacteria. Strains of Bacteroides amylophilus attached to starch granules in levels from 0% to 60% of the bacteria in the original attachment solution (Minato & Suto, 1979). A variety of different polymers and other materials from hydrophobic (PTFE) to hydrophilic (glass) were used to investigate bacterial attachment (Pringle & Fletcher, 1983b). Each bacterial species was found to attach in different levels to individual surfaces. The results from these studies and the results presented in Table 2.1 and 2.2 show that the individual nature of a bacterium is of prime importance in attachment and that generic classification bears no relationship to bacterial attachment.

Among the bacterial characteristics which may be important in the attachment of bacteria to solid surfaces are the bacterial motility; bacterial cell surface characteristics; and physiological activity. In the results described earlier in this Chapter both motile and non-motile bacteria were capable of high and low attachment levels, indicating flagella may not play a dominant role in attachment. Bacterial motility will increase the likelihood of a bacterium encountering the surface and may raise the kinetic energy of a bacterium, sufficient to overcome electrostatic repulsion barriers between the

bacterial surface and the solid surface which normally bear like-charges (Chapter 5). A marine Pseudomonas sp, was shown to attach in greater numbers to polystyrene surfaces when flagella were present than when the flagella had been artificially removed (Fletcher, 1980a). The effect was not found to be the result of lowering the number of collisions with a substratum by deflagellation, but rather because the force of contact was lowered which in turn would lower the likelihood of the bacterium overcoming an electrostatic repulsion barrier.

Flagella may have a more direct role in attachment, acting as the point of initial contact between the bacterium and the solid surface. A flagellum has a narrower diameter than a bacterium's cell body, which will result in the lowering of the repulsion barrier with a solid surface (Rogers, 1979). The smaller potential energy barrier between a substratum and flagellum may result in the kinetic energy of locomotion becoming sufficient to overcome the barrier as indicated by Fletcher (1980) and may allow a flagellum to approach a surface closely enough to interact with it. The flagellum bridges the gap between the solid surface and the cell body which may then react itself with the substratum. Sjoblad & Doetsch (1982) demonstrated that a flagellum could in fact be the initial point of contact for polarly flagellated bacteria, e.g. Vibrio cholerae, though the cell body also adsorbed to the glass surfaces.

Bacterial motility may then be important in the attachment of some bacteria, but it does not represent the only component in the interaction nor always the major influence as indicated by results described in Section 2.4. The bacterial cell surface is a major component in the attachment interaction between a solid surface and a bacterial cell, since it represents the point of contact between the two. The range of attachment levels described for the selected species in this Chapter may be partly due to different cell surface characteristics

and therefore different physico-chemical interactions with the solid surface (Chapters 4 and 5). Some indications of an interrelationship between physiological activity and permanent bacterial attachment may be shown in the results described in this Chapter, since there are considerable differences in levels of attachment between buffer and nutrient. The relationship between attachment and activity may be direct, i.e. attachment being an active process, or indirect, i.e. the metabolic activity of the bacteria influencing certain bacterial characteristics e.g. cell wall composition. The effect of bacterial activity on attachment may vary with species. There are, however, other explanations for two different levels of attachment between buffer and nutrient observed in this Chapter, i.e. physico-chemical interactions (Chapter 6). The question of a physiological role in attachment is dealt with in depth in Chapters 4 and 7.

The variability of bacterial cell surfaces and physiological activity may be the explanation for changes in the attachment levels of individual bacterial species between experiments, shown in the attachment results presented in Table 2.1 and 2.2. Slight changes in the growth phase in batch culture may produce quite large changes in these two factors. The growth phase of a bacterium in batch culture has previously been shown to have a large influence on attachment to solid surfaces. A marine Pseudomonas sp. attached in largest numbers when in the late lag phase/early stationary growth stage (Fletcher, 1977). Several other workers have shown such effects (Zvyagintsev, 1973; Minato & Suto, 1979) and results for bacterial isolates in this thesis show similar changes (Chapter 7). The variability in bacterial cell surface characteristics and resultant changes in bacterial attachment levels is discussed in Chapter 4.

Although bacterial characteristics represent a major component in the physico-chemical adsorption of bacteria onto solid surfaces (Chapter

5), two further factors can influence attachment, the solid surface and the liquid phase.

The characteristics of the solid substratum had a major influence on the numbers of bacteria attaching (Table 2.1 and 2.2). The nature of the solid surface can be described in terms of hydrophobicity (Chapter 5, Sections 5.2 and 5.6.3). High energy reactive surfaces are hydrophilic in nature, e.g. glass, whereas low energy unreactive surfaces are hydrophobic, e.g. PTFE. It has been found that the hydrophobicity of a substratum has an effect on bacterial attachment (Fletcher & Loeb, 1979; Pringle & Fletcher, 1983b). Investigations into the interactions of a marine Pseudomonas sp. with a variety of hydrophobic plastics and hydrophilic metals showed that attachment levels changed with solid substratum (Fletcher & Loeb, 1979). Similarly, by treating glass with water-repellant coating DiSalvo and Daniels (1975) showed that estuarine bacteria attached less strongly to water-repellant glass than clear glass. The results presented in Table 2.1 and 2.2 similarly show changes in levels of bacterial attachment with different surfaces, i.e. PD and TCD surfaces. It is, however, impossible to generalize as to which substratum favours bacterial attachment since it varied with species. A further complicating factor in the interaction between bacterium and substratum is the nature of the surrounding liquid phase. This is clearly demonstrated by the attachment results by bacteria in buffer and nutrients (Table 2.1 and 2.2). The presence of nutrients modifies levels of bacterial attachment to solid surfaces, the effect varying with the nature of the substratum and the particular bacterial species involved (Table 2.1 and 2.2).

The influence of organic molecules on bacterial attachment to surfaces has long been known. As early as 1959 Zvyagintsev observed that the adsorption capacity of a number of bacteria was changed with the cleanliness of a glass substratum in respect to organic matter (Zvyagintsev, 1959). A medium filtrate was found to differentially affect

the attachment of a marine Pseudomonas sp, to surfaces raising it to polystyrene and lowering to platinum (Fletcher & Loeb, 1979), showing the influence of mixed organic molecules on levels of bacterial attachment as indicated by the results in Table 2.1 and 2.2.

Several workers have shown that a variety of pure proteins can impair, raise, or leave unaltered the attachment of a range of bacterial species to solid surfaces. Bovine serum albumin, gelatin, fibrinogen and pepsin inhibited the attachment of a marine Pseudomonas sp. to polystyrene surfaces, while protamine sulphate and histone were found to have no effect (Fletcher, 1976). The attachment of Aeromonas liquifaciens, Escherichia coli and Pseudomonas fluorescens was found to increase in the presence of casein and gelatin and decrease when either protamine sulphate or albumin was present in the liquid phase (Meadows, 1971).

The effect of organic macromolecules on bacterial attachment is the result of two factors. First, macromolecules tend to accumulate at a solid/liquid interface, producing a conditioning film over the surface (Chapter 6). Both the solid surface and the bacterial cell surface may have conditioning films and these may alter the characteristics that each surface presents to the surrounding environment.

The interaction between the solid and bacterial surfaces may be considerably modified by such conditioning films, altering levels of attachment. Each bacterium or solid surface, depending on its particular characteristics, may accumulate different conditioning films. Thus a range of interactions are possible depending on the bacterial species or bacterial variability, and the type of solid surface (Table 2.1 and 2.2, Chapter 6).

Secondly, organic molecules will alter the liquid surface tension which in turn will influence bacterial adsorption to solid surfaces (Chapters 5 and 6).

Bacterial attachment to solid surfaces is based on a tripartite interaction between the substratum, the liquid phase and the bacterium itself. The interactions are complex and susceptible to even small changes in any of the three components involved in the reaction. Since a bacterium is physiologically active and capable of responding to environmental changes, it may represent the most variable factor in bacterial adhesion.

2.6 SUMMARY

1) The extent of bacterial attachment to polystyrene solid surfaces was different for each bacterial species.

2) The generic classification of the isolated bacteria had no relationship to their abilities to attach to solid surfaces.

3) Bacterial motility, although of significance in attachment, was not a prime determinant of bacterial adhesion to solid surfaces. Both motile and non-motile bacteria showed low and high attachment levels. Bacterial physiological activity and cell surface characteristics may be of importance in attachment interactions.

4) Bacteria attached in different levels to the hydrophobic (PD) surface and the hydrophilic (TCD) surface. Some bacteria attached in greatest numbers to the PD surface, others to the TCD surface and still others attached in equal numbers to both. Since bacterial attachment is dependent upon physico-chemical interactions, e.g. hydrophobic and charge interactions, different types of interactions occur with different surfaces thus causing changes in levels of bacterial attachment.

5) The attachment interactions between bacteria and solid surfaces were altered by the presence of nutrients in the liquid phase. Attachment levels could be either raised, lowered or unaltered, attachment varying independently with the bacterium and the surface. The presence of organic molecules acts by producing conditioning films on the solid and bacterial surfaces which will alter their surface

characteristics. Macromolecules will also change the surface tension of the liquid phase which will influence attachment.

CHAPTER THREE

THE ATTACHMENT OF A MIXED BACTERIAL POPULATION

3.1 AIMS

To compare the attachment of a mixed bacterial community with that of its individual members in pure culture.

3.2 INTRODUCTION

In natural aquatic environments populations of bacteria will consist of a range of species, and it is from this mixed population that bacteria attach. It is difficult to extrapolate attachment interactions of pure bacterial cultures to those of bacterial communities, therefore bacterial attachment within mixed populations is worthy of study.

Bacteria within a mixed population will be in competition, for nutrients etc, and may therefore exert a considerable influence on each other. However, interactions between bacteria are not only physiological in nature but also physico-chemical in that bacterial flocs form in the liquid phase (Harris & Mitchell, 1973; Atkinson & Daoud, 1976). It may be the case that the attachment of individual species of bacteria will be affected by the presence of other bacterial species.

The attachment of a known mixed population of bacteria, which consisted of the Chromobacterium sp, P. fluorescens, P. aeruginosa and E. cloacae, to PD and TCD surfaces was investigated and compared with that of pure cultures of the individual members of the community. The community and individual species were grown under the same conditions in continuous culture prior to attachment, and the attachment interactions with the solid substrata occurred under identical conditions.

3.3 MATERIALS AND METHODS3.3.1 Culture Apparatus and Conditions

A continuous culture system was developed which allowed the maintenance of constant temperature and aeration of bacterial cultures at constant dilution rates (Fig. 1). A culture volume of 800 mls was aerated with filtered (Glass wool) water saturated air and stirred to ensure good mixing of the culture. The medium consisting of 54.4g

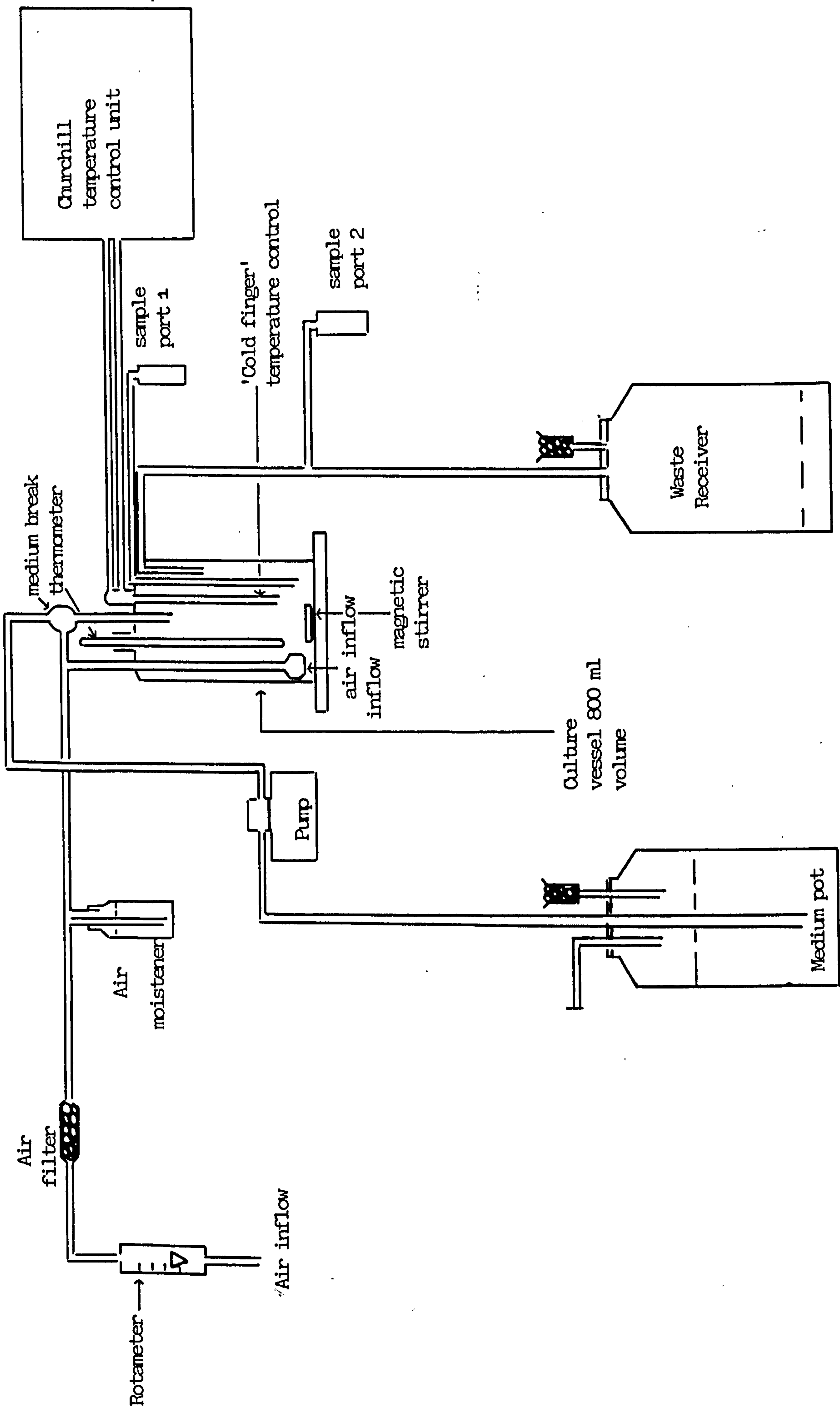


Figure 3.1 The continuous culture system (not drawn to scale)

KH_2PO_4 (Fisons Scientific Reagents, Loughborough); 10g peptone (Oxoid Ltd, London); 7g yeast extract (Lab M, London) in 10 litres of distilled water adjusted to pH 7.4 with 1M NaOH, was supplied to the cultures in all experiments at a dilution rate (D) of 0.1 h^{-1} . An 800 ml volume of medium was allowed to equilibrate to a constant temperature of 15°C ($\pm 2^\circ\text{C}$), maintained by a Churchill Chiller Thermo Circulator (Churchill Ltd England) and to a steady aeration before inoculation of test organisms.

3.3.2 Organisms and Inoculation Procedure

(a) Mixed Culture: The thirteen identified bacteria (Chapter 2) were each grown in 100 ml PYE broth medium (Section 2.3.2) on an orbital incubator at 150 rpm at 15°C for 16 hrs. One ml quantities of each culture were then aseptically inoculated into an equilibrated chemostat. The resultant culture was allowed to grow in batch at a constant temperature (15°C) and aeration for 24 hrs before turning on the flow and establishing the dilution rate of 0.1 h^{-1} . After 6 days the community was sampled, the chemostat refilled and the mixed culture allowed to grow for 6 hrs before re-establishing the original dilution rate. Growth was continued for a total of 14 days from the onset of continuous culture.

(b) Pure Cultures: Individual organisms isolated and identified from the mixed continuous culture at 6 days were each grown in PYE broth culture (Section 2.3.2) for 24 hrs. Pure chemostat cultures were then established by aseptically inoculating 1 ml aliquots of the individual broth cultures into equilibrated chemostats. The cultures were allowed to grow for 24 hrs before the dilution rate was established. A minimum of 3 medium volume changes in the culture vessel were allowed before the bacterial culture was sampled. The bacteria investigated in pure culture were Pseudomonas fluorescens (H_2), Pseudomonas aeruginosa (H_{15}), Enterobacter cloacae (H_{12}) and a Chromobacterium sp (H_{31}).

3.3.3 Attachment Assay

100 ml volumes of culture were collected from the chemostat and centrifuged at 10,950 av.g (Beckman model 5-21B centrifuge). The cells

were washed once in 0.01M HEPES buffer (Sigma Chemical Company, Poole), pH 7.4, to remove any contaminating nutrients and resuspended in 0.01M HEPES buffer, pH 7.4 and PYE medium broth (Section 2.3.2) to an optical density of 0.1 at 540 nm in a colorimeter (Corning Colorimeter 252). Cell counts were made of each resuspended sample using a counting chamber (Weber Scientific Int. Ltd, Lancing, Sussex). The attachment assay procedure has been described (Section 2.3.4).

The duplicate plates, both polystyrene petri dishes (PD) and tissue culture dishes (TCD), were however assayed at time periods of 5, 15, 30 and 60 mins for each set of samples.

3.3.4 Species numbers and Identification of the Mixed Community

The proportions of individual members of the mixed community were estimated by plating dilutions (10^{-7} , 10^{-9} and 10^{-11}) of the chemostat culture onto a duplicate series of different agar media. The media used were PYE medium agar (Section 2.3.2), nutrient agar (Lab M, London) King's B media (Section 2.3.3.4b) and MacKonkey agar (Oxoid Ltd, London) (for demonstration of lactose fermentation). Using these media individual species were differentiated by pigment production, the fermentation of lactose and colony description. An estimate of species numbers was made from the dilution plates, and the community composition was described in terms of the percentage of the total numbers represented by each species. Individual colony types were identified by the API 20E system (API Laboratory Products Ltd, Basingstoke) (described in Section 2.3.3b).

3.3.5 Identification of Bacteria Attached to PD and TCD Surfaces

Attachment assays were run on PD and TCD plates for the mixed cultures in both buffer and nutrients, using the attachment assay procedure (Section 2.3.5) washing with 0.01M HEPES (Sigma Chemical Company, Poole), pH 7.4, to remove loosely attached cells, and then the duplicate surfaces were swabbed sterily. These specimens were then inoculated and streak spread onto a duplicate series of the media (Section 3.3.4).

Individual colonies were visually identified and checked through the API 20E system (API Laboratory Products Ltd, Basingstoke).

3.4 RESULTS

One organism, a Chromobacterium sp, predominated over the other bacterial species (Pseudomonas fluorescens, Pseudomonas aeruginosa and Enterobacter cloacae) in the mixed continuous culture (Table 3.1). The proportion of the community represented by the Chromobacterium sp, differed very little between the 6 day and the 14 day incubation periods. However, the two Pseudomonas sp. showed an increase in numbers, replacing the E. cloacae which was totally removed from the liquid phase at 14 days.

It is apparent from the attachment results of the pure bacterial culture (Figs. 3.2, 3.3, 3.4, 3.5) that each isolate was capable of different levels of attachment to PD and TCD. The influence of surface type and buffer or nutrient on adhesion was different for each species. The rates of attachment, i.e. the increase with time, was also different for each bacterium, varying between rapid rises in levels of attachment for the Chromobacterium sp (Fig. 3.2) to no change in adhesion with time for Pseudomonas fluorescens (Fig. 3.3). The rates were changed occasionally by the presence or absence of nutrients, e.g. the presence of nutrient caused rapid rises in the attachment of Pseudomonas aeruginosa to both PD and TCD compared to the buffered system (Fig. 3.4).

It is apparent from Table 3.2 and Figures 3.2 to 3.7 that the attachments of the bacterial community, both the 6 day and 14 day sample, were not related to any individual member of the population. The attachment to PD and TCD in buffer and nutrient did not correspond to that of the predominant organism, Chromobacterium sp, which represented approximately 64% of the total population, (Table 1), nor any of the other bacteria in the community. Similarly, the change in attachment with time found for the mixed bacterial population was not related to

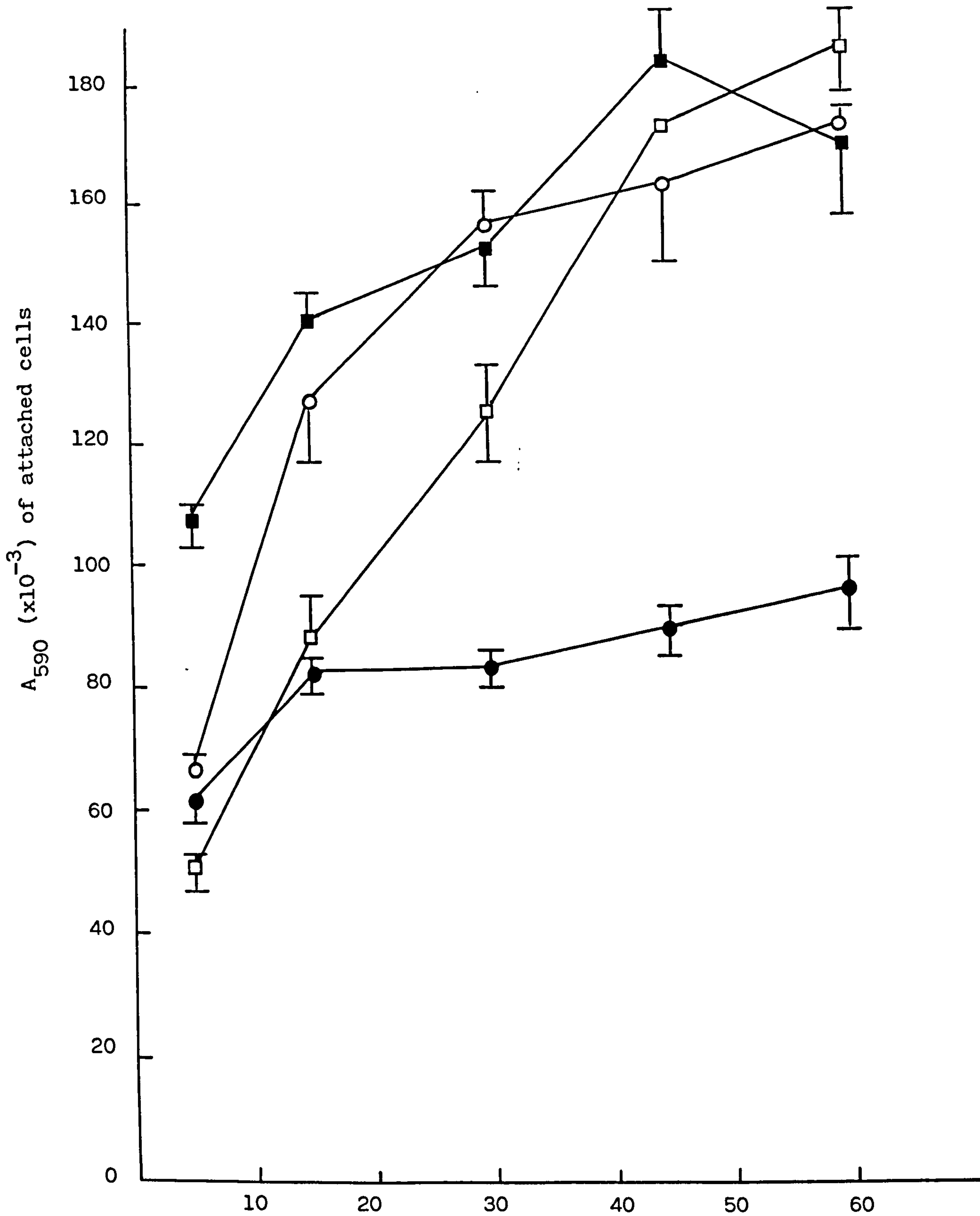


Figure 3.2 The attachment of a Chromobacterium sp grown in continuous culture ($D = 0.1h^{-1}$).

(○), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (□), cells attached in PYE to PD; (■), cells attached in PYE to TCD

The bars represent the 95% confidence limits of the mean ($n = 8$)

$A_{590} (\times 10^{-3})$ of
attached cells

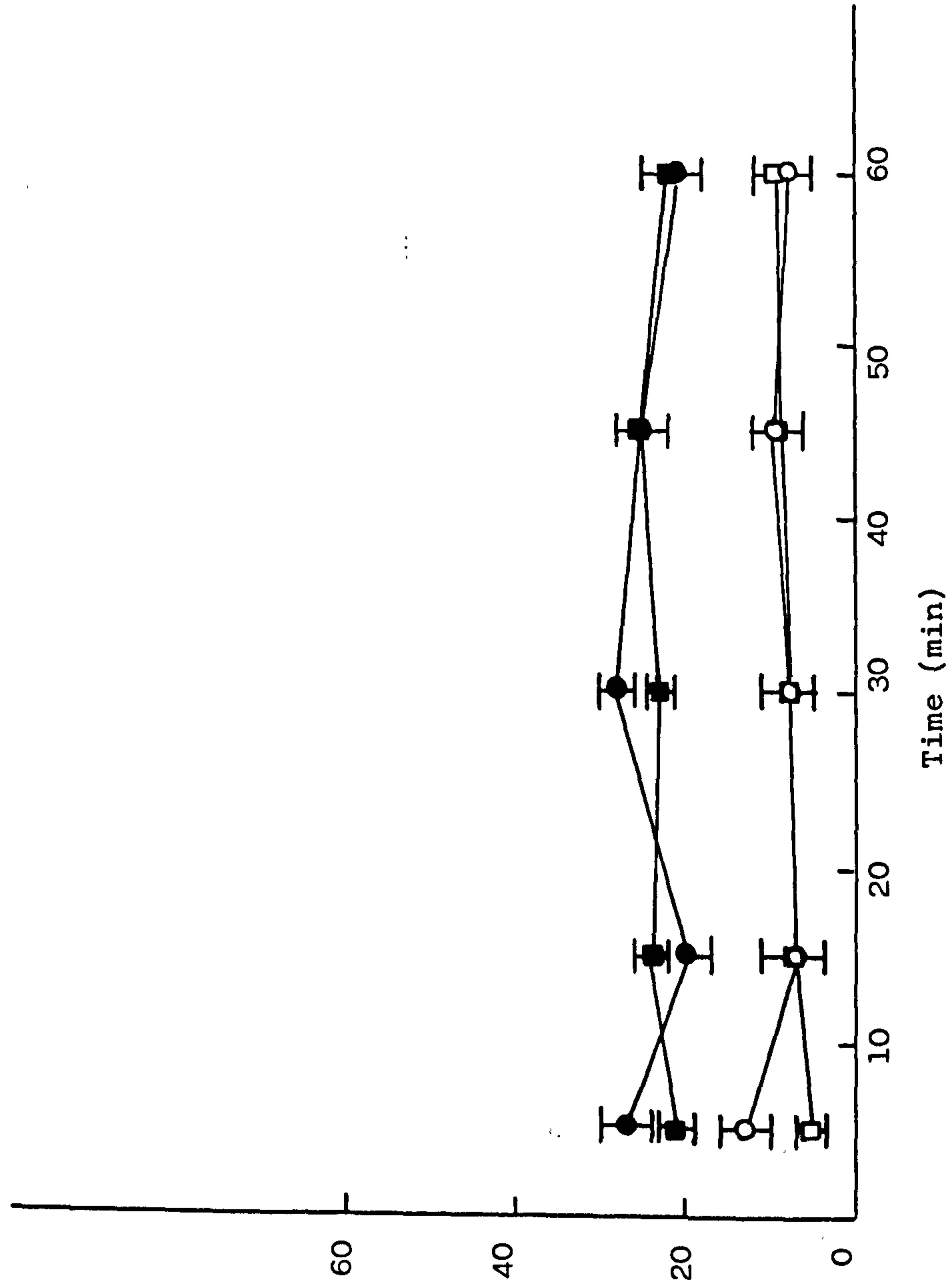


Figure 3.3 The attachment of Pseudomonas fluorescens grown in continuous culture ($D = 0.1h^{-1}$) (○), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (■), cells attached in PYE to PD; (□), cells attached in PYE to TCD.

The bars represent the 95% confidence limits of the mean ($n = 8$)

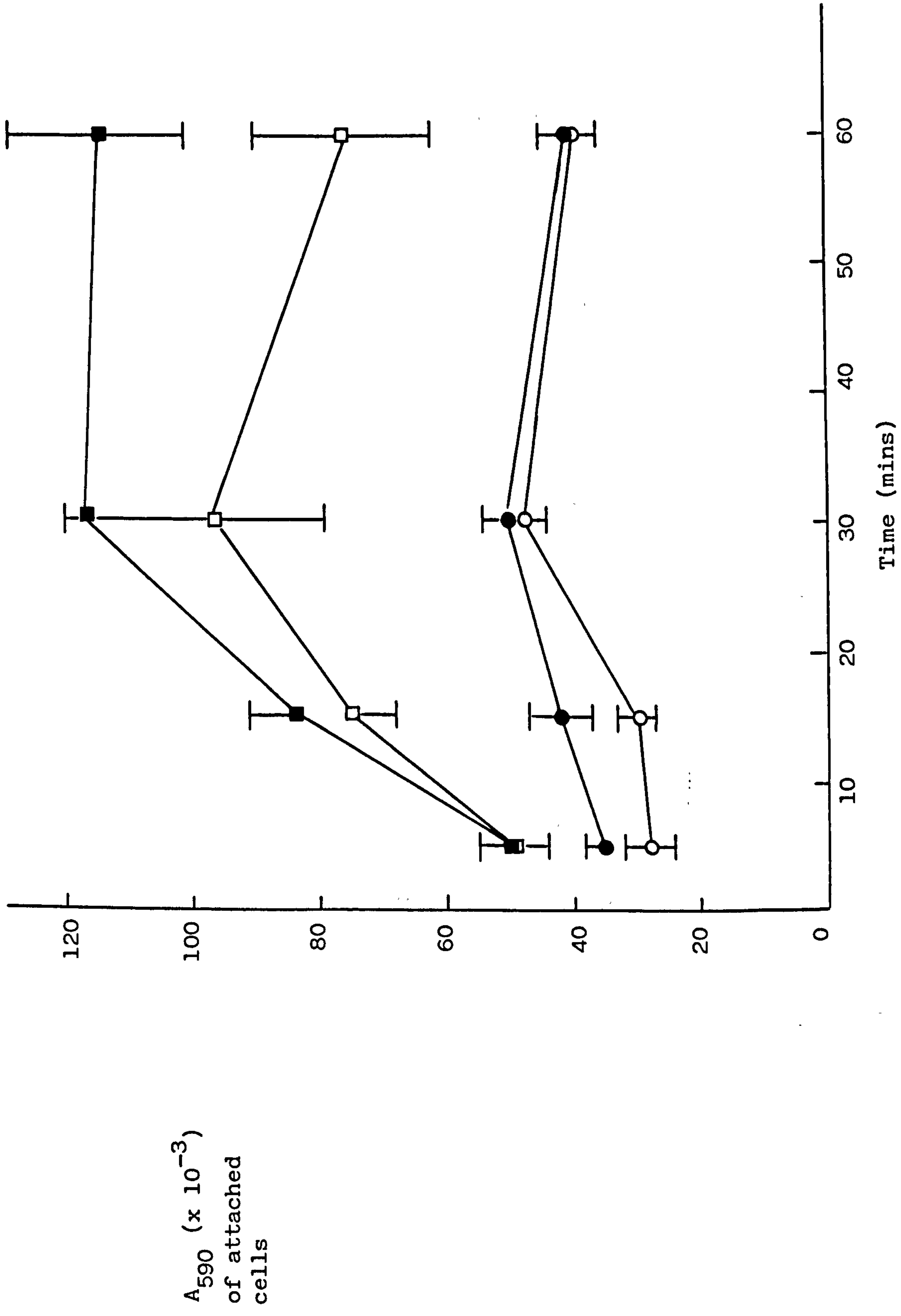


Figure 3.4 The attachment of *Pseudomonas aeruginosa* grown in continuous culture ($D = 0.1h^{-1}$) (O), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (□), cells attached in PYE to PD; (■), cells attached in PYE to TCD.

The bars represent the 95% confidence limits of the mean ($n = 8$)

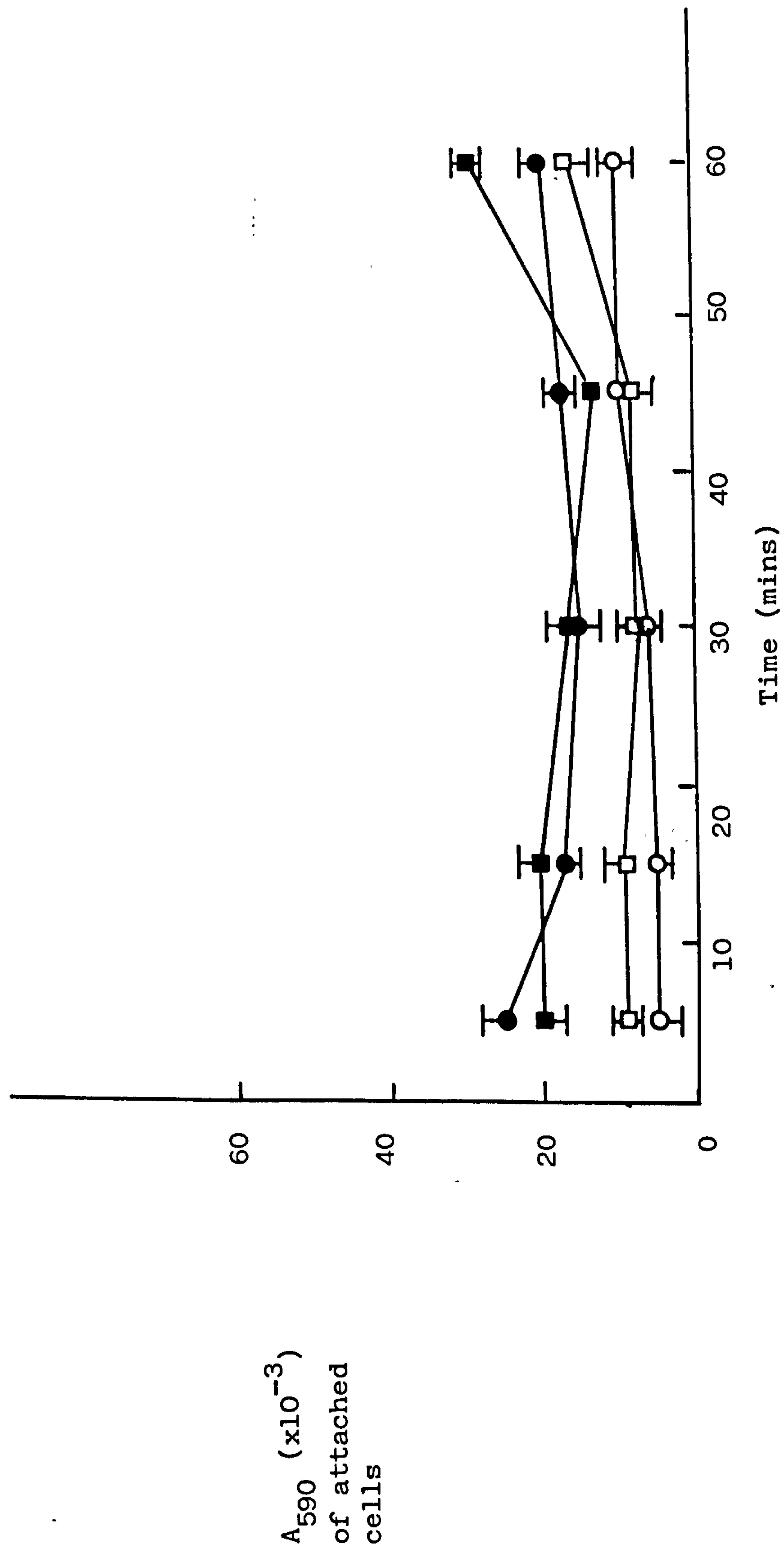


Figure 3.5

The attachment of Enterobacter cloacae grown in continuous culture ($D = 0.1h^{-1}$) (O), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (□), cells attached in PYE to PD; (■), cells attached in PYE to TCD. The bars represent the 95% confidence limits of the mean ($n = 8$)

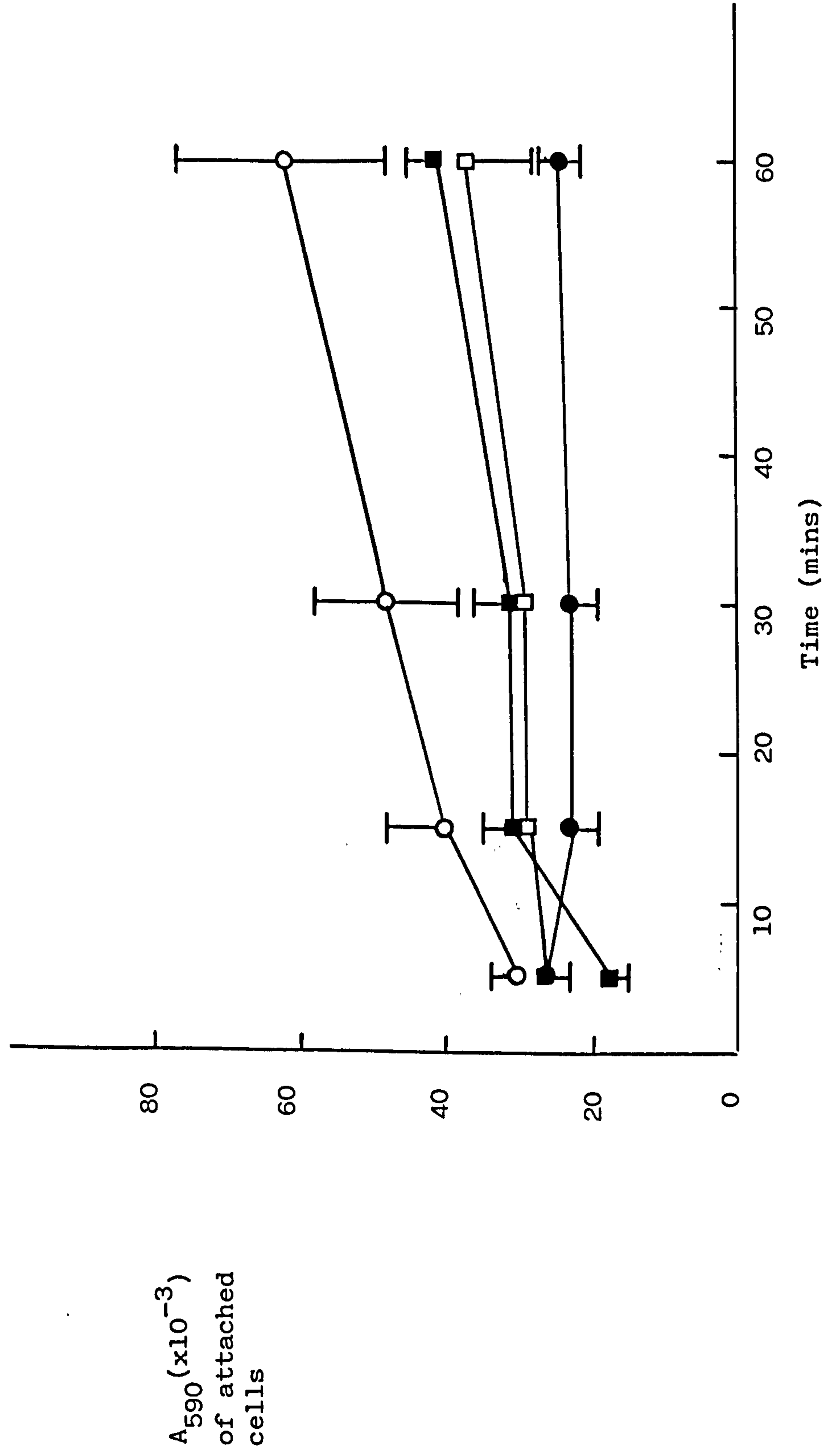


Figure 3.6 The attachment of a mixed bacterial community grown in continuous culture for 6 days ($D = 0.1h^{-1}$)

(O), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (□), cells attached in PYE to PD; (■), cells attached in PYE to TCD.

The bars represent the 95% confidence limits of the mean ($n = 8$)

A_{590} ($\times 10^{-3}$)
of attached
cells

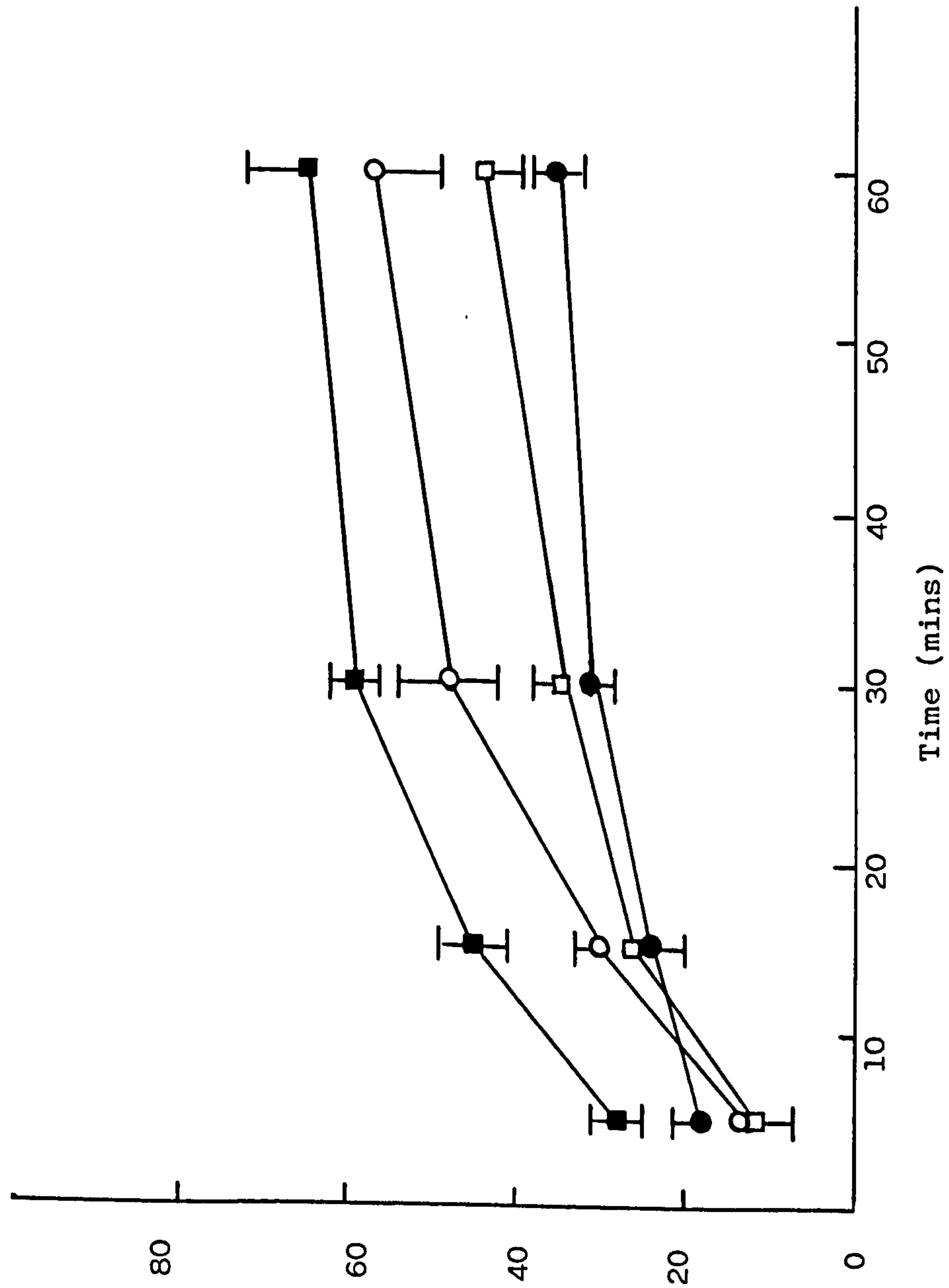


Figure 3.7 The attachment of a mixed bacterial community grown in continuous culture for 14 days ($D = 0.1h^{-1}$) (O), cells attached in HEPES buffer to PD; (●), cells attached in HEPES buffer to TCD; (□), cells attached in PYE to PD; (■), cells attached in PYE to TCD. The bars represent the 95% confidence limits of the mean ($n = 8$)

the changes shown by any individual species.

The differences in the community of bacteria between the 6 day and 14 day samples, e.g. the loss of E. cloacae, may partially have been responsible for the change in the attachment interactions of the community. There was an increase in bacterial adhesion to the TCD surface in both buffer and nutrient (Fig. 3.6 and 3.7, Table 3.2), and also a more rapid rise in the attachment, up to 30 mins with the sample taken after 14 days culture incubation. In all the experiments involving mixed bacterial cultures, each species represented in the liquid phase was isolated from each attachment substratum (Table 3.3). There was no attempt to estimate the numbers of individual species attached to the solid surfaces because of the difficulty of quantitatively removing the bacteria either by physical or chemical methods. However, the predominant microorganism in the liquid phase, and the bacterium capable of the highest attachment levels, i.e. the Chromobacterium sp. predominated on the test surfaces.

Comparison of the final attachment levels, i.e. at 60 mins, between the community and the pure cultures is misleading, since the numerical levels of each bacterium in the community was less than those in the attachments of individual species (Table 3.4). However, the proportion of the community represented by the Chromobacterium sp. was high. Given the cell numbers of the Chromobacterium sp. in the mixed culture attachments (Table 3.4) and its high levels of attachment in pure culture (Fig. 3.2) predicted levels of attachment for the communities would be of at least double those found (Fig. 3.6 and 3.7). This indicates that the Chromobacterium sp, may be prevented from attaching to its full potential.

3.5 DISCUSSION

The community showed very different attachment characteristics to those of any of its component parts. This indicates the possibility

TABLE 3.1 The Species composition of a Mixed Bacterial Community
grown in continuous culture ($D = 0.1 \text{ h}^{-1}$) for 6 days
and 14 days

Species	% of total population from 6 day culture	% of total population from 14 day culture
Chromobacterium sp	64.7	63.6
Pseudomonas fluorescens	24.1	29.1
Pseudomonas aeruginosa	5.2	7.3
Enterobacter cloacae	6.0	0

TABLE 3.2 The attachment of mixed bacterial populations to two
polystyrene surfaces after 1 hr attachment in buffer or
nutrient

	Attachment ($A_{590} \times 10^{-3}$)		Attachment ($A_{590} \times 10^{-3}$)	
	PD	Surface	TCD	Surface
	Buffer	Nutrient	Buffer	Nutrient
6 day	62 (± 13) ^a	37 (± 6)	24 (± 3)	41 (± 3)
14 day	57 (± 8)	44 (± 5)	35 (± 3)	65 (± 7)

^a Parenthetical values represent the 95% confidence limits of the mean
($n = 8$)

TABLE 3.3 The presence or absence of bacterial species in a Liquid Phase and attached to the PD and TCD surfaces from 6 day and 14 day mixed continuous culture

Species	6 DAY MIXED CULTURE			14 DAY MIXED CULTURE		
	Liquid Phase	PD Surface	TCD Surface	Liquid Phase	PD Surface	TCD Surface
Chromobacterium sp	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Enterobacter cloacae	+	+	+	-	-	-

TABLE 3.4 Bacterial cell numbers in pure culture attachment experiments
and the community attachment experiments

Attachment species or community	Organisms/ml at OD 0.1 (540 nm) in the attachment solutions	Calculated ^a individual species ml ⁻¹ at OD 0.1 (540 nm) in the 6 DAY COMMUNITY	Calculated ^a individual species ml ⁻¹ at OD 0.1 (540 nm) in the 14 DAY COMMUNITY
Chromobacter-ium sp.	3.5 x 10 ⁸	2.9 x 10 ⁸	2.54 x 10 ⁸
Pseudomonas fluorescens	4.0 x 10 ⁸	1.1 x 10 ⁸	1.16 x 10 ⁸
Pseudomonas aeruginosa	4.2 x 10 ⁸	2.3 x 10 ⁷	2.9 x 10 ⁷
Enterobacter cloacae	4.5 x 10 ⁸	2.7 x 10 ⁷	0
6 day community	4.5 x 10 ⁸	-	-
14 day community	4.0 x 10 ⁸	-	-

^a $\frac{\text{total organisms ml}^{-1} \text{ in community at OD 0.1 (540 nm)} \times \% \text{ species A in community (see Table 3.1)}}{100}$

100

= organisms/ml species A in OD 0.1 (540 nm) attachment solution of the community

that interactions between the bacteria themselves may influence attachment in the community. It is possible to visualize a series of such affects:

- 1) interactions between different species in the bulk liquid phase, e.g. competition between organisms and species aggregation
- 2) the production of substances, e.g. polymers (Chapter 4) by one or more of the bacteria which may affect levels of attachment
- 3) competition for attachment sites
- 4) the displacement of reversibly attached bacteria by another species approaching the surface
- 5) attached bacteria may exert a 'zone of influence' in their immediate area on a solid surface, by some as yet unknown mechanism, which could inhibit or promote the attachment of other bacteria.

Bacterial cells of similar and dissimilar species can interact in a complementing or opposing manner. Physiological competition between species may result in changes in the capability of bacteria to attach to solid surfaces. It has long been known that bacteria can physically interact in the liquid phase forming microbial flocs (Harris & Mitchell, 1973). The process of floc formation is partly dependent on a series of biological factors such as the microbial concentration and the floc concentration. It should be emphasized that the presence of other microorganisms or strains can influence floc formation (Atkinson & Daoud, 1976). Thus normal processes of competition within communities are supplemented by important physico-chemical interactions which may influence the attachment of individual species to solid surfaces, as well as to each other.

The production of metabolites, enzymes, or polymers by bacteria will affect bacterial adhesion, and in a mixed culture there is potential for greater variation. The importance of these substances in attachment was demonstrated for a marine Pseudomonas sp. (Fletcher & Marshall, 1982). Bubble contact angles, a technique involving measuring the contact angle of air bubbles on test substrata suspended over a chamber filled with liquid, were measured using two bacterial supernatants as conditioning

films. The first supernatant was obtained by centrifuging a bacterial culture (S1), the second by suspending cells from a centrifuged culture and re-centrifuging the cells (S2). It was found that the effect of the adsorbed supernatants on bubble contact angles varied with substratum, showing little effect on a polystyrene surface (PD), and considerable effect on a tissue culture polystyrene surface (TCD). The S1 supernatant was shown to inhibit the attachment of the Pseudomonas sp, to TCD. The same marine Pseudomonas sp, attached less to platinum, but more to polystyrene in the presence of another medium filtrate (Fletcher & Loeb, 1979).

Several workers have indicated the diverse effects of various protein molecules on the attachment of bacteria (Fletcher, 1976; Meadows, 1971). The data presented in this Chapter and Chapter 2 shows the large effect on bacterial attachment of PYE, which consists of a complex array of organic molecules including peptides and amino acids. Its presence can inhibit or promote attachment (Chapter 2 and Section 3.3. above) as occurs with pure proteins (Meadows, 1971; Fletcher, 1976) and similar interactions can be visualized for macromolecules produced by the community. The effect of PYE on bacterial attachment varies with species (Table 2.1, 2.2 and Figs. 3.2 and 3.5) suggesting a similar difference in the effects of molecular products of the community on the attachment of individual species.

Solid surfaces may present a limited number of potential points for bacterial attachment and monolayer formation. It may be possible that one organism may saturate the attachment sites on a substratum, thereby preventing the adhesion of a second bacterial species or strain, but the evidence for this is contradictory. Resin particles saturated with Bacillus prodigiosus so they could adsorb no more cells of this organism, could adsorb a quantity of Bacillus mycoides, though in reduced amounts as compared to adsorption of a pure culture (Zvyagintsev, 1962). Similar work using soil particles indicated that soil saturated

by Serratia marcescens prevented the adsorption of a second organism Bacillus cereus var mycoides (Chudiakow, 1926, cited in Marshall 1971). The evidence then points to a degree of competition for attachment sites on surfaces between species, perhaps dependent on the nature of the solid surface and the bacterium involved. There is also an indication that different bacteria, to some extent, may interact with different attachment sites (Zvyagintsev, *et al.*, 1962).

Since all the bacteria of the community were present on the solid substrata in these experiments, there was no evidence that one particular organism saturated the surface attachment sites.

The ability of one bacterial species to displace an already adsorbed species has been investigated by several workers. Bacterial interactions with soil particles have demonstrated the possibility of sorption exchange when large numbers of Serratia marcescens were added to limas mud from a salt lake (Rubentschik, *et al.* 1936, cited in Marshall, 1971). The results indicated that one cell desorbed for every additional ten cells of S. marcescens sorbed. The possibility exists that a similar interaction may occur between different species and not just within species. Zvyagintsev (1962); however, found that bacteria adsorbed onto anionite AN-2F and cationite CDB-J resin particles could only show adsorption exchange with a second species if they were loosely attached and easily removed by washing. Those cells which attached firmly could not be displaced.

Thus, there is the possibility that adsorption exchange may influence the attachment of the mixed population with more weakly attached species desorbing from the surface.

Once the bacteria are attached to a solid substratum, the nature of the surface in their immediate vicinity will be changed. This effect would be due to differences in physico-chemical parameters, e.g. surface

tension, and because of biological factors, e.g. enzyme production and polymer production. A bacterium may, therefore, produce a 'zone of influence' on the surface. The effects of this may be to enhance or inhibit attachment of fresh bacteria to the area and may be particularly important in mixed community attachment.

The succession in bacterial biofilm development has been well established for both marine and freshwater habitats (Section 1.3). In the later stages of bacterial film development, after the initial colonization by rod-shaped copiotrophic bacteria (Chapter 1), secondary bacterial colonizers invade. These stalked or budding microorganisms, e.g. Caulobacter, Hyphomicrobium sp, attach to the developing biofilm between 2 and 5 days (Corpe, 1973; Marshall et al. 1971). It has been suggested that such succession is dependent on changes in conditions caused by the primary colonizers (Corpe, 1973). These changes may relate to the release of nutrients, surface active agents or polymeric materials by the attached bacteria which enhance the attachment of the stalked and budding microorganisms (Marshall et al. 1971). Although such interactions are determined by well developed bacterial films, the monolayer of cells produced by one hour attachment, may have similar effects, e.g. the presence of polymeric material.

All the bacterium-bacterium interactions suggested above are potentially involved in the attachment processes of a mixed bacterial population. The relative importance of each factor may change with the species present in the community, and the nature of the liquid and solid phases. To these interactions should be added the normal physico-chemical determinants (Chapter 5) of attachment, which would have their influence on the adhesion of individuals within the community. The changes in attachments found between the 6 and 14 day communities underline the variability and complexity of attachment in a mixed population, and may alter the successional development of a biofilm.

3.6 SUMMARY

1) Individual bacterial species isolated from a mixed culture demonstrated different levels of attachment, which were independently affected by the nature of the solid substratum and the liquid phase.

2) When mixed communities attached to PD and TCD in buffer and nutrient the attachment levels did not reflect those of the predominant species, a Chromobacterium sp, nor of any other species in the population.

3) Changes in the community composition were reflected in different attachment patterns for the mixed population.

4) Each species of the suspended community was represented on the attachment surfaces under all experimental conditions. This included Enterobacter cloacae which had low numbers in the community and low attachment levels in pure culture.

5) A series of interactions that would affect bacterial attachment within a community were proposed;

i) interaction between different species in the bulk liquid phase

ii) the production of macromolecules, e.g. enzymes, polymers, by individuals in the community which affect attachment

iii) a competition for attachment sites

iv) the displacement of an attached bacterium by another species approaching the surface

v) a 'zone of influence' exerted by attached bacteria in their immediate vicinity, altering physico-chemical and biological parameters and thus affecting the attachment of bacteria approaching the surface.

CHAPTER FOUR

THE EFFECT OF GROWTH RATE, NUTRIENT SOURCE AND CARBON/NITROGEN RATIO
ON BACTERIAL CELL SURFACE CHARACTERISTICS AND BACTERIAL ATTACHMENT TO
SOLID SURFACES4.1 AIMS

To determine the variability of bacterial cell surface characteristics and bacterial attachment to solid surfaces for bacteria cultured in different carbon/nitrogen ratio media, carbon source media, and at a range of growth rates in continuous culture.

4.2 INTRODUCTION

The cell walls of bacteria have been shown to alter with changes in environmental conditions, e.g. with variations in growth rate and growth limiting substrate (Tempest & Ellwood, 1969, cited in 1972). Similarly exopolysaccharide production can change both in the quantity of exopolymer formed and in its constituent molecules with growth conditions and growth rate in chemostat cultures (Wilkinson, 1958; Williams & Wimpenny, 1977). Since bacterial attachment is probably predominantly determined by interactions between the cell wall and the solid surface, it is important to establish how variable the cell surfaces of bacteria are and if such variations are reflected in levels of attachment.

In order to determine the variability of bacterial cell surface characteristics, bacteria were grown at a range of dilution rates in continuous culture; in different nutrient sources; and in different carbon/nitrogen ratio media, both in batch and continuous culture.

P. fluorescens, E. cloacae, the Chromobacterium sp, and the Flexibacter sp, were then investigated for cell surface characteristics, such as hydrophobicity and charge, by hydrophobic interaction chromatography and electrostatic interaction chromatography respectively. Less specific

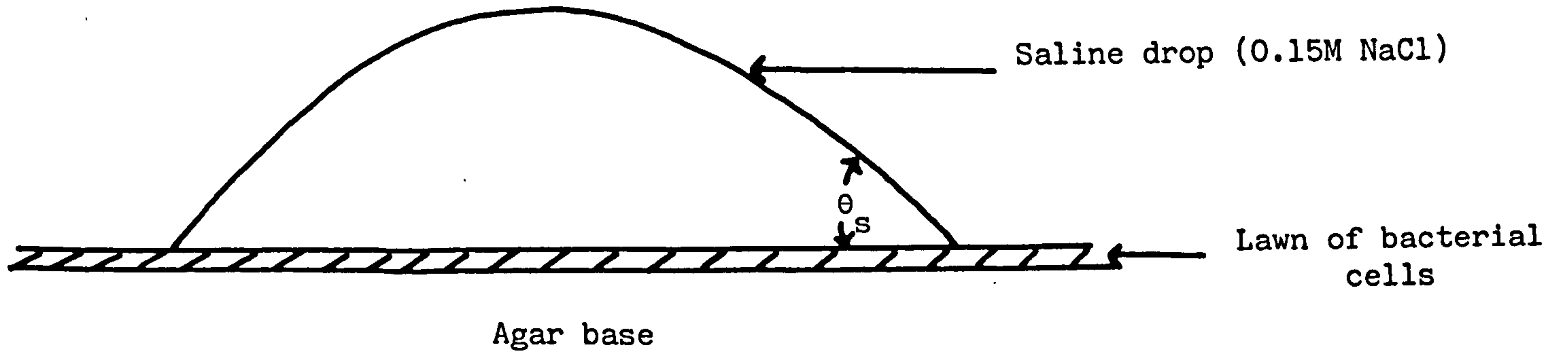


Figure 4.1 Diagrammatic representation to show the contact angle (θ_s) of 0.15M NaCl solution on a lawn of bacterial cells supported by an agar base

changes were determined by saline contact angle (θ_s) measurements on lawns of bacterial cells, i.e. the angle of contact between a drop of saline and the cells (Fig. 4.1). The more hydrophobic the lawn of cells the larger θ_s . Changes in bacterial surface characteristics were then related to levels of attachment to PD and TCD surfaces. When investigating the effect of bacterial growth rate on attachment both the level and the rate of attachment was considered.

4.3 MATERIALS AND METHODS

4.3.1 Organisms

Four bacterial species were selected for investigation on the basis of particular characteristics which might affect their attachment to solid surfaces and the nature of their cell surface. The selected bacteria and principal criteria in their selection were:

- 1) Pseudomonas fluorescens (H_2); no flocculation during growth in PYE medium broth (Section 2.3.2) (Appendix Table 1);
- 2) Enterobacter cloacae (H_{12}); flocculation during growth in PYE medium broth (Appendix Table 1);
- 3) Chromobacterium sp, (H_{31}); flocculation during growth in PYE medium broth (Appendix Table 1);
- 4) Flexibacter sp, (H_{38}); no flocculation during growth in PYE medium broth, but capable of gliding motility across a solid substratum (Appendix Table 1).

4.3.2 Culture Conditions and Inoculation Procedure

(a) Batch Culture:

The bacteria were cultured in a selection of media containing different carbon sources and carbon/nitrogen ratios. The 100 ml glucose minimal medium described in Section 2.3.4 was adapted for each nutrient condition. The autoclaved buffer (KH_2PO_4) (15 min, $121^\circ C$) and filter sterilized salt solution (0.45 μm cellulose-acetate filter) was as described in Section 2.3.4 (the pH was again adjusted to 7.4 with 1M NaOH). The carbon substrate and concentrations of carbon and nitrogen source were, however, varied - the result was eight different media

consisting of:

- 1) 0.05g glucose (Fisons Scientific Reagents, Loughborough), 0.38g NH_4Cl (Fisons Scientific Reagents, Loughborough) per 100 mls (carbon-limited);
- 2) 0.2g glucose, 0.02g NH_4Cl per 100 mls (nitrogen limited);
- 3) 0.2g glucose, 0.38g NH_4Cl per 100 mls (carbon/nitrogen sufficient);
- 4) 0.2g glucose, 0.38g NH_4Cl , 10% (v/v) glycerol (Fisons Scientific Reagents, Loughborough) per 100 mls;
- 5) 1% (w/v) lactose (Fisons Scientific Reagents, Loughborough), 0.38g NH_4Cl per 100 mls;
- 6) 1% (w/v) galactose (BDH Chemicals Ltd, Poole), 0.38g NH_4Cl per 100 mls;
- 7) 1% (w/v) mannose (Sigma Chemical Company, Poole), 0.38g NH_4Cl per 100 mls;
- 8) 1% (w/v) sucrose (May & Baker Ltd, London), 0.38g NH_4Cl per 100 mls.

The carbohydrates and NH_4Cl were autoclaved separately (10 min, 121°C) and added aseptically in the required concentration to the basal medium.

Pure bacterial cultures were grown in these media and stored at 4°C . 1 ml aliquots of the stock cultures were inoculated into identical fresh media and incubated at 15°C on a rotary incubator at 150 rpm for 24 hrs before sampling. The Chromobacterium sp, was sampled after 24 hrs and also 48 hrs incubation.

b) Continuous Culture:

i) PYE medium.

The continuous culture apparatus described in Section 3.3.1 was used to grow each of the four bacteria in pure culture in buffered PYE medium (Section 3.3.1). The continuous culture vessel was aseptically inoculated

from a pure PYE broth culture grown at 15°C for 24 hrs. The bacterium was then grown for 24 hrs in the chemostat in batch culture, before establishing the dilution rate as described in Section 3.3.2b. The dilution rate for each of the pure bacterial cultures was changed successively over the series of rates, 0.05 h^{-1} , 0.1 h^{-1} , 0.15 h^{-1} and 0.2 h^{-1} . The cultures were only sampled after a minimum of three complete volume changes at each dilution rate.

ii) Minimal media.

A second series of growth conditions were established in continuous culture at a dilution rate of 0.025 h^{-1} . Pure bacterial cultures were grown in three carbon/nitrogen ratios using a minimal medium. The medium consisted of $54.4\text{g KH}_2\text{PO}_4$ (Fisons Scientific Reagents, Loughborough) in 10 litres of distilled water adjusted to pH 7.4 with 1M NaOH solution (BDH Chemicals Ltd, Poole) and sterilized by autoclaving (40 min, 121°C). Aseptically added to this was 60 ml of a filter sterilized (0.45 cellulose-acetate filter) salt solution consisting of $10\text{ g l}^{-1}\text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough); $1.0\text{ g l}^{-1}\text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$ (May & Baker Ltd, London); $0.4\text{ g l}^{-1}\text{ FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough); and $0.1\text{ g l}^{-1}\text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Fisons Scientific Reagents, Loughborough). Selected concentrations of filter sterilized (0.45 cellulose-acetate filter) glucose and NH_4Cl were then aseptically added to complete the medium. The final concentrations of glucose and NH_4Cl were:

- 1) 0.5 g l^{-1} glucose, $3.8\text{ g l}^{-1}\text{ NH}_4\text{Cl}$ (carbon limited)
- 2) 2 g l^{-1} glucose, $0.2\text{ g l}^{-1}\text{ NH}_4\text{Cl}$ (nitrogen limited)
- 3) 2 g l^{-1} glucose, $3.8\text{ g l}^{-1}\text{ NH}_4\text{Cl}$ (carbon and nitrogen sufficient)

Each bacterial species was grown in pure culture in the glucose minimal medium described in Section 2.3.4 for 24 hrs at 15°C on an orbital incubator rotating at 150 rpm before adopting the inoculation and growth procedure described above (Section 3.3.2b).

c) Agar Culture:

To observe bacterial growth and the extent of polymer production, subjectively estimated, on different carbon source and carbon/nitrogen ratio media, pure cultures of each bacterial species were grown on minimal medium agar plates. The media consisted of 1% (w/v), ionagar (Oxoid Ltd, London); 5.4g KH_2PO_4 in 100 mls of distilled water adjusted to pH 7.4 by 1M NaOH. This was sterilized by autoclaving (15 mins, 121°C) cooled and 0.6 mls of filter sterilized (0.45 cellulose acetate filter) salt solution (Section 4.3.4b) was aseptically added to the agar. The different carbon sources and nitrogen source (described in Section 4.3.2a) were autoclaved (15 mins, 121°C) separately and added to the minimal media to the concentrations and ratios described above (Section 4.3.2a).

4.3.3 Attachment Assays

The batch cultures and 100 ml samples from the chemostat cultures were centrifuged at 10,960 av.g (Beckman model J-21B centrifuge). The cells were washed once in 0.01M HEPES buffer (Sigma Chemical Company, Poole), pH 7.4, to remove contaminating nutrients, and resuspended in 0.01M HEPES buffer, pH 7.4, to an optical density of 0.1 at 540 nm in a colorimeter (Corning Colorimeter 252).

The attachment assay has been described in Section 2.3.4, and the procedure remained unaltered. However, the times that duplicate plates of PD and TCD were assayed were extended for some of the experiments up to a range of 5, 15, 30 and 60 mins.

4.3.4 Contact Angle Measurements on Lawns of Cells

A method of measuring the contact angle (θ) on lawns of bacterial cells was developed based on the method of van Oss et al. (1975).

Bacterial cells were harvested from minimal media batch cultures by centrifugation at 10,960 av.g (Beckman model J-21B centrifuge) and washed once in 0.15M NaCl solution (Fisons Scientific Reagents, Loughborough).

The cells were resuspended to a concentration of approximately 10^{10} cells/ml in 2 mls of 0.15M NaCl to obtain a thick slurry of cells. Drops of the cell suspension were then placed on an agar base which consisted of 2% ionagar (Oxoid Ltd, London) and a 10% (w/v) glycerol (Fisons Scientific Reagents, Loughborough) in 0.15M NaCl solution autoclaved at 121°C , 15 mins. The agar base was prepared by covering a glass slide in a petri dish with a layer of agar approximately 2 mm thick. Once the agar solidified the agar coated slide was removed with a scalpel. The cell slurry was spread across the agar base to give a covering of cells, and air dried. After approximately 2 hrs drying at room temperature the cell lawn took on a matt appearance and contact angle measurements were initiated.

The advancing contact angle of a 10 μl drop of 0.15M NaCl solution (θ_s) was measured every 10 mins, within 10 secs of drop application, using a vernier microscope with a goniometer eye piece (Pti, Liss, Hampshire). Duplicate measurements were made at each 10 min interval and were considered reliable if they did not vary by more than 0.5° . No two measurements were made on the same area of cells. The values of θ_s changed with time until a plateau was reached where the values remained relatively constant with time (varying approximately $\pm 1^{\circ}$); this plateau was maintained between 30 and 45 mins. An average value of measurements during this period was taken to represent the saline contact angle of the lawn of cells (van Oss et al. 1975).

4.3.5 Hydrophobic Cationic and Anionic Interaction Chromatography

The method for investigating the hydrophobic, cationic and anionic interaction of different bacterial cells was adapted from the methods of Dahlbäck et al., (1981) and Pederson (1980).

Pasteur pipettes (John Poulter Ltd, Essex) were plugged with glass wool to a depth of 1 mm. The pipettes were then washed with 95% ethanol (J. Burrough Ltd, London) followed by 0.1M NaCl solution (Fisons

Scientific Reagents, Loughborough). Phenyl-Sepharose CL-4B gel (Sigma Chemical Company, Poole) for hydrophobic interaction chromatography (HIC) was diluted 1:1 with 0.1M NaCl and degassed for up to 10 mins under a vacuum pump before being packed into the pipettes to a depth of 8 mm. 1 ml quantities of either anion exchange resin (Dowex 1 x 8 mesh size 100/200; 80-150 μm) (Sigma Chemical Company, Poole) or cation exchange resin (Dowex 50wx8 mesh size, 100/200; 80-150 μm) (Sigma Chemical Company, Poole) were suspended in 1 ml of 0.1M NaCl and degassed before packing into the Pasteur pipettes to a depth of 8 mm. These two types of columns were used to investigate electrostatic interaction chromatography (EIC). Both the HIC and EIC columns were washed through with 5 mls of 0.1M NaCl and were maintained at 4°C saturated with 0.1M NaCl for up to 72 hrs. (The stored HIC and EIC columns adsorption capacity was tested by comparing the adsorption of cells on stored and fresh columns, and was found to be unaffected by storage).

The affinity of the bacterial species after growth at different dilution rates in continuous culture for the hydrophobic gel and the electrostatic resins was determined by sampling 100 mls of culture from the chemostat, centrifuging the cells once at 10,960 av.g (Beckman J-21B centrifuge) and resuspending the cells in 20 mls KH_2PO_4 buffer (5.4 gl^{-1} , pH 7.4). The cells were then incubated at 15°C for 2 hrs after the addition of 1 $\mu\text{Ci ml}^{-1}$ ^3H L-leucine (Radiochemical Centre, Amersham) (leucine could be assimilated by the four bacteria investigated). The ^3H -labelled bacteria were then washed 3 times at 10,960 av.g (Beckman model J-21B centrifuge) with 0.1M NaCl to remove unmetabolized label and resuspended in 0.1M NaCl solution to a volume of 10 mls, final cell density was approximately 10^9 cells/ml. The cells were suspended in a high ionic strength solution to promote hydrophobic interactions.

One ml volumes of the cell suspension were applied to duplicate columns of the hydrophobic gel, the anionic resin and the cationic resin, followed by a 3 ml quantity of 0.1M NaCl. The columns were washed a further two times with 4 mls 0.1M NaCl. The eluate from all three applications was collected separately.

Quadruplicate 100 μ l quantities from each eluate and the original cell suspension were individually counted for radioactivity, using 10 mls of a scintillation cocktail consisting of 2:1 (v/v) toluene (Fisons Scientific Reagents, Loughborough): Triton X-100 (Sigma Chemical Company, London) and 0.6% (w/v) of the scintillator 2-(4'-tert-butylphenyl)-5-(4"-biphenyl-1-3',4,-oxadiazole) (butyl PBD; Fisons Scientific Reagents, Loughborough). Counts were made with a liquid scintillation counter (System LS-7000, Beckman Instruments Inc, Fullerton, Calif.) using glass vials. The extent of quenching was determined by the H number system, an external standard monitoring system based on the Compton electron spectrum generated by a 137 gamma Cs source (Horrocks, 1977). The counts were repeated if there was discrepancy > 5% between the quadruplicate values. The final eluate had counts normally not much above background level. The extent of leakage from the labelled cells was determined by counting 100 μ l duplicate quantities of the final wash supernatant, a level of 10% leakage was the maximum considered acceptable.

The results were expressed in terms of the percentage of counts remaining in the column, i.e. total counts from the applied cells minus total counts from the 3 eluates divided by the total counts multiplied by 100. The results from duplicate columns were considered mutually consistent if they varied by no more than 5% in the estimate of counts remaining in the column, any greater discrepancy and the experiment was repeated. The higher the percentage of cells remaining in the HIC and EIC columns, the greater the hydrophobic or electrostatic interactions, respectively.

Table 4.1 provides a summary of the experimental design to determine

TABLE 4.1 The Experimental Design to Determine the Effect of Growth Conditions and Growth Rate on Bacterial Cell Surface Characteristics and Attachment Levels

EXPERIMENTAL PROCEDURES	GROWTH CONDITIONS		BATCH CULTURES		CONTINUOUS CULTURE	
			Carbon/Nitrogen Ratio Media	Carbon Source Media	Pye (D=0.05,0.1,0.15,0.2h ⁻¹)	Carbon/Nitrogen Ratio Media (D=0.025 h ⁻¹)
θ_s measurements and 60 minute attachment assay			^a +	+	-	-
			(Figs. 4.2-4.6)	(Figs.4.2-4.6)		
HIC and EIC measurements and 5 and 60 min attachment assay			-	-	+	-
					(Tables 4.7 - 4.10)	
5,15,30,45 and 60 min attachment assays			-	-	+	-
					(Figs. 4.7-4.10)	
60 min attachment assay			+	-	-	+
			(Tables 4.3 - 4.6)			(Tables 4.3 - 4.6)

^a

the experimental procedures relate to results shown in the Figures and Tables enumerated

the effect of culture conditions and growth rates on bacterial cell surface characteristics and on bacterial attachment.

4.4 RESULTS

4.4.1 The Effect of Growth Conditions on Bacterial Attachment and Cell Surface Characteristics

Bacterial growth in different carbon/nitrogen ratio and carbon source media had a profound effect on the subsequent attachment of bacteria to a hydrophobic polystyrene surface (PD) and the more hydrophilic tissue culture dish surface (TCD).

The four bacterial species investigated, Pseudomonas fluorescens, Enterobacter cloacae, a Chromobacterium sp, and a Flexibacter sp, all reacted differently in their attachment with changes in culture conditions in batch culture (Fig. 4.2-4.6; Appendix Tables 6-8). The variations in adhesion were, on occasions, very large, e.g. Pseudomonas fluorescens showed very high attachment in glucose with glycerol and relatively low attachment in the remaining culture conditions (Fig. 4.2). Similar differences were found for all the species; however, the different bacteria did not show the same response to any particular carbon source or carbon/nitrogen ratio condition. The organism least susceptible to large changes in attachment after growth in different nutrient conditions was the Flexibacter sp, (Fig. 4.6). The Chromobacterium sp, showed considerable differences in attachment after growth in the media for 24 hrs and after growth for 48 hrs, e.g. after growth for 24 hrs in glucose and nitrogen sufficient medium attachment was approximately a third of that after 48 hrs growth (Fig. 4.4. and 4.5).

Attachment levels to the PD and TCD surfaces were independently variable with the culture conditions, e.g. Enterobacter cloacae showed attachment after growth in galactose approximately four times higher to the TCD surface than to the PD surface, whereas after growth in sucrose

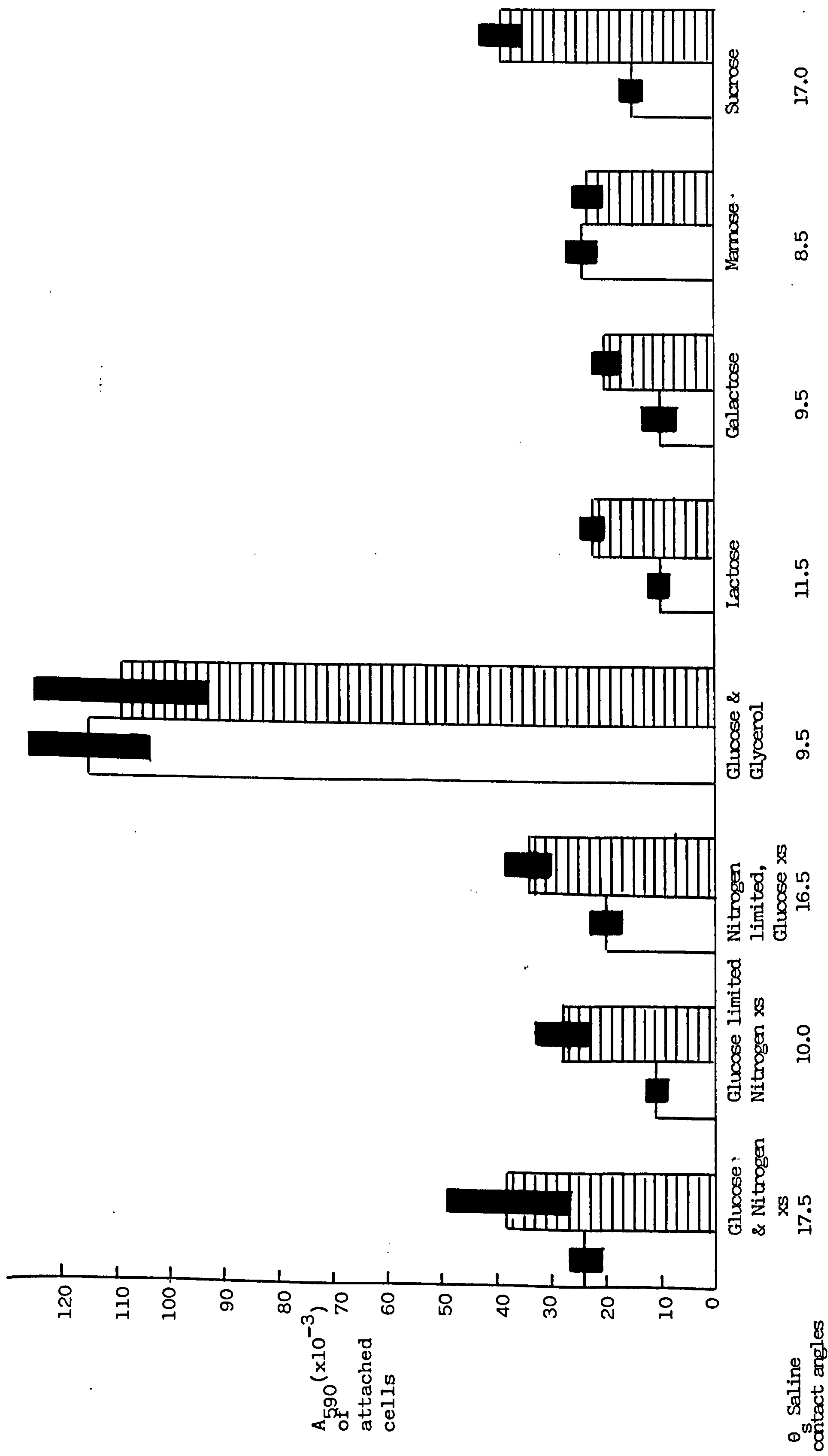


Figure 4.2 The effect of carbon/nitrogen ratio and carbon source on the cell surface characteristics (measured by contact angles) and attachment of *Pseudomonas fluorescens* (60 minutes attachment)

(\square), cells attached to the PD surface; (\blacksquare), cells attached to the TCD surface
 (), 95% confidence limits of the mean (n = 8)

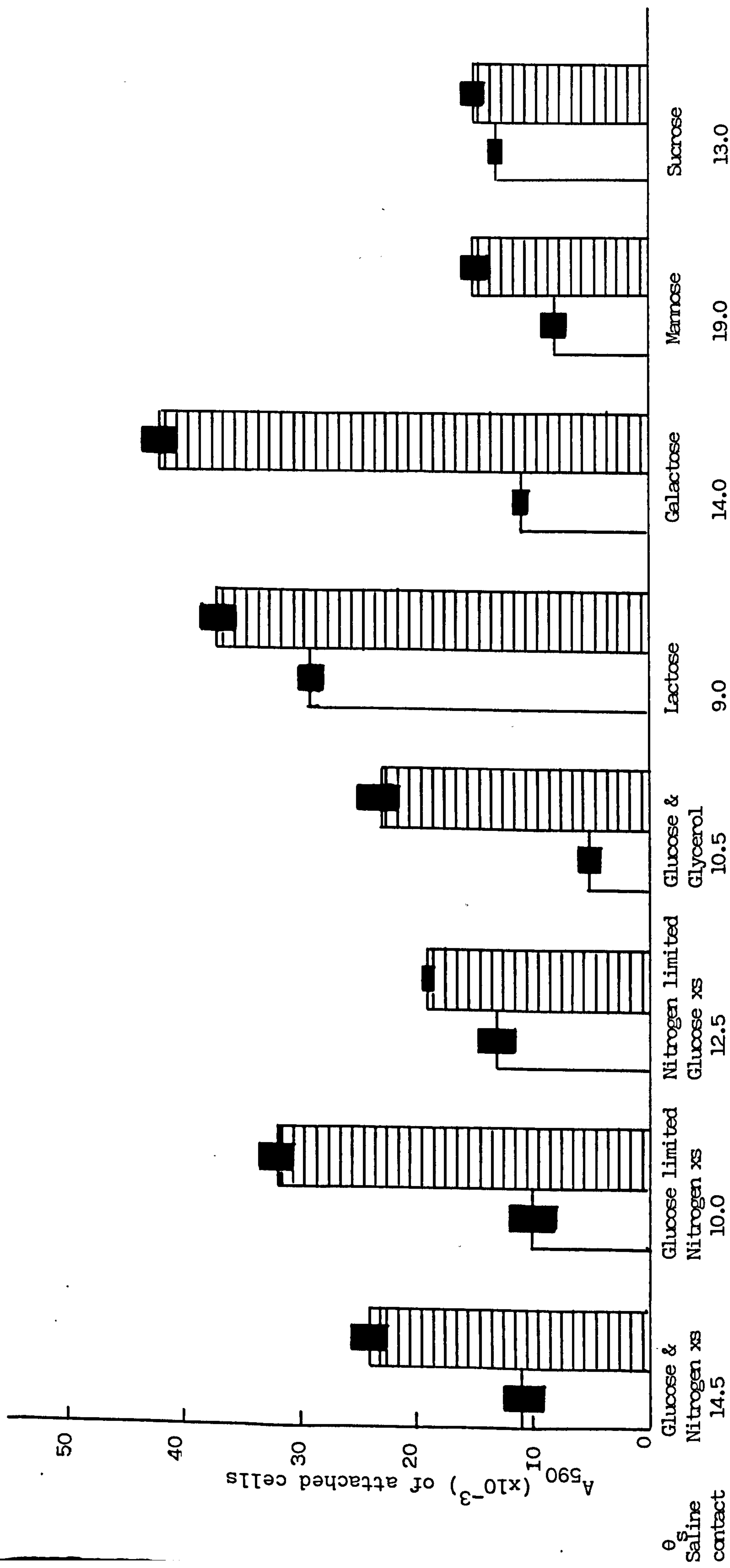


Figure 4.3 The effect of carbon/nitrogen ratio and carbon source on the cell surface characteristics (measured by contact angles) and attachment of *Enterobacter cloacae* (60 minutes attachment)

(□), cells attached to PD surface; (■), cells attached to TCD surface; (■), 95% confidence limits of the mean (n = 8)

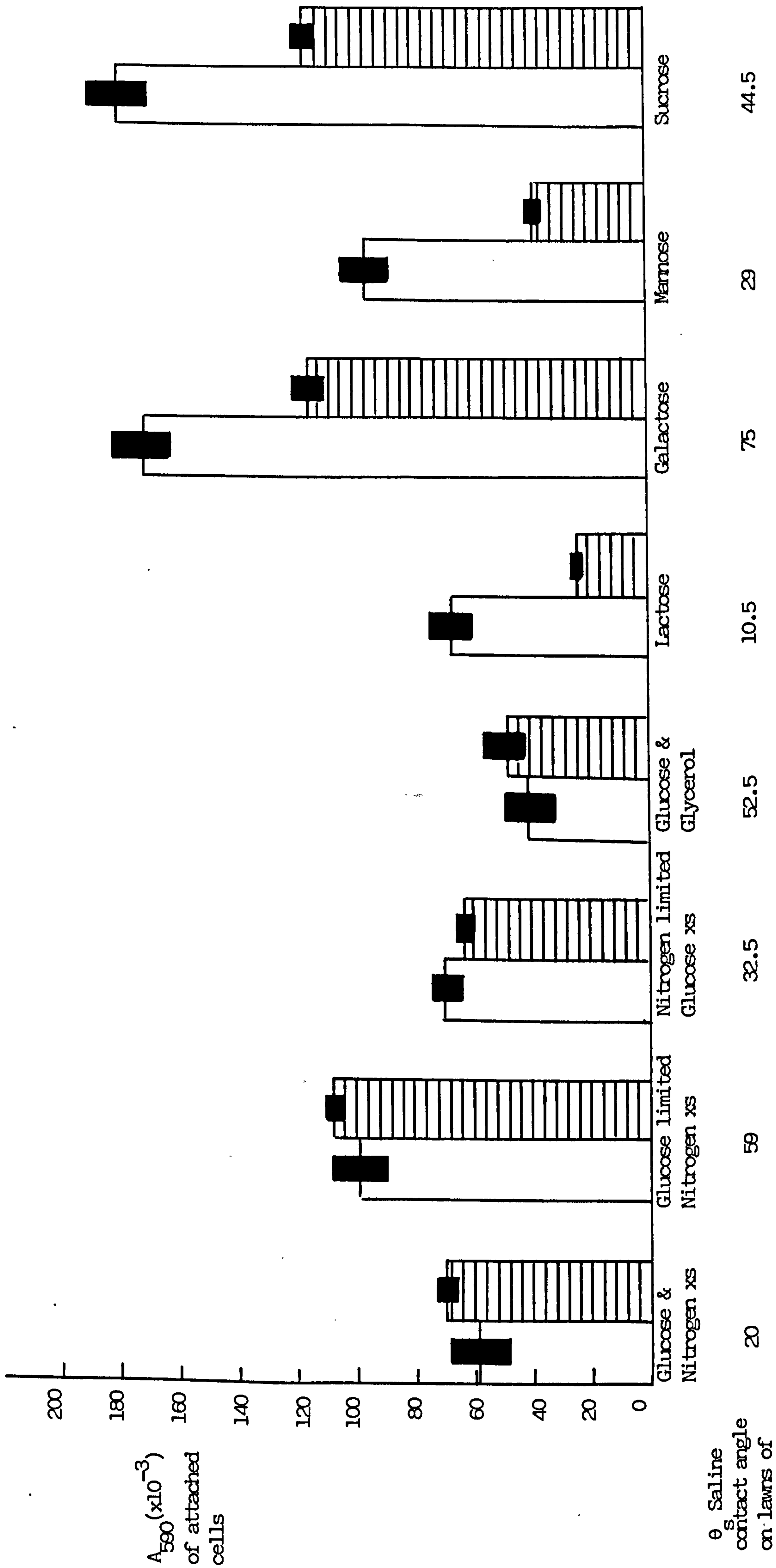


Figure 4.4 The effect of carbon/nitrogen ratio and carbon source on the cell surface characteristics (measured by contact angles) and attachment of the *Chronobacterium* sp after 24 hrs growth (60 minutes attachment)

(\square), cells attached to the PD surface; (▨), cells attached to the TCD surface; (\blacksquare), 95% confidence limits of the mean ($n = 8$)

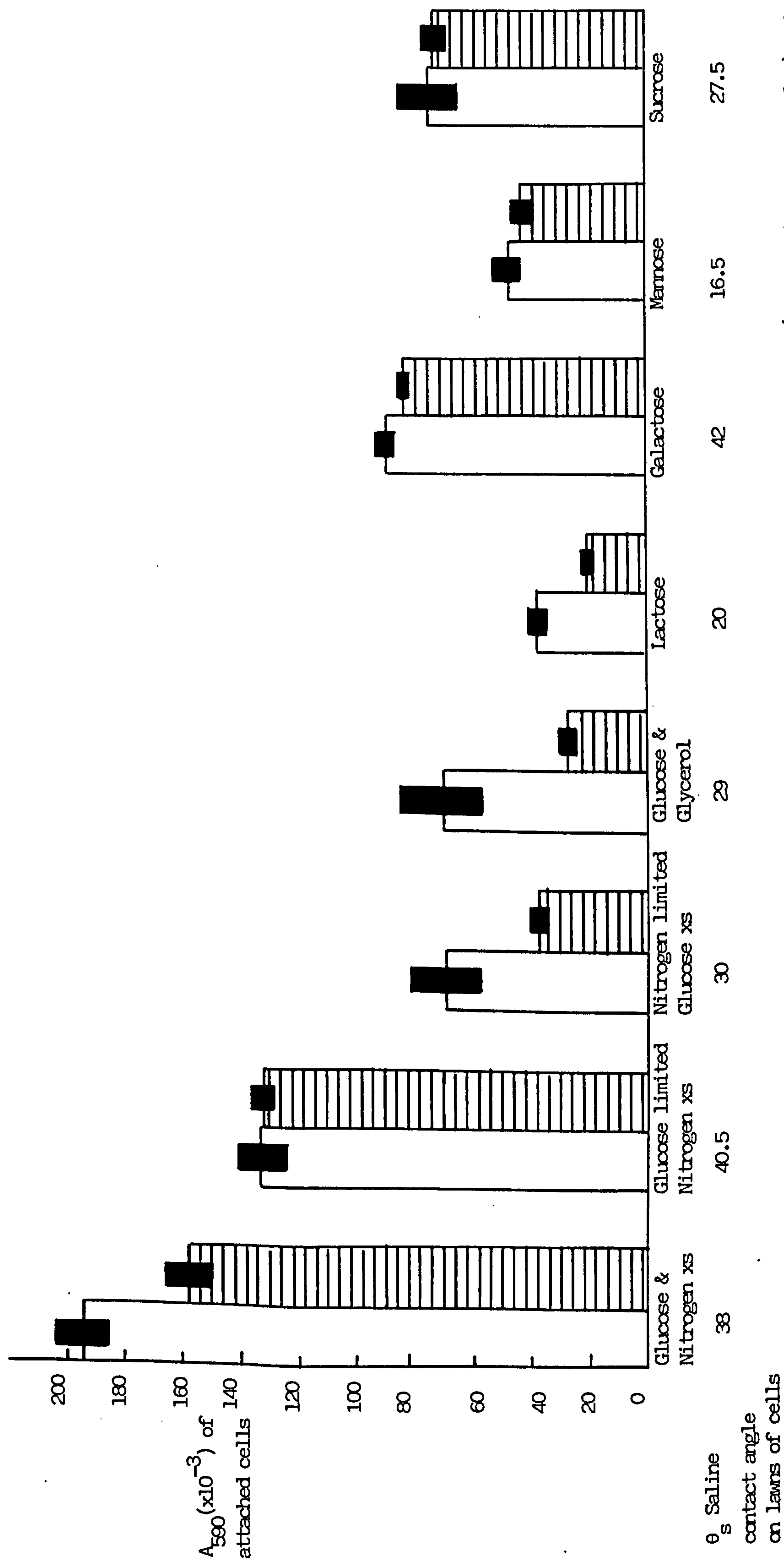


Figure 4.5 The effect of carbon/nitrogen ratio and carbon source on the cell surface characteristics (measured by contact angles) and attachment of *Chromobacterium* sp after 48 hrs growth (60 minutes attachment)

(\square), cells attached to PD surface; (\blacksquare), cells attached to TCD surface; (\blacksquare), 95% confidence limits of the mean ($n = 8$)

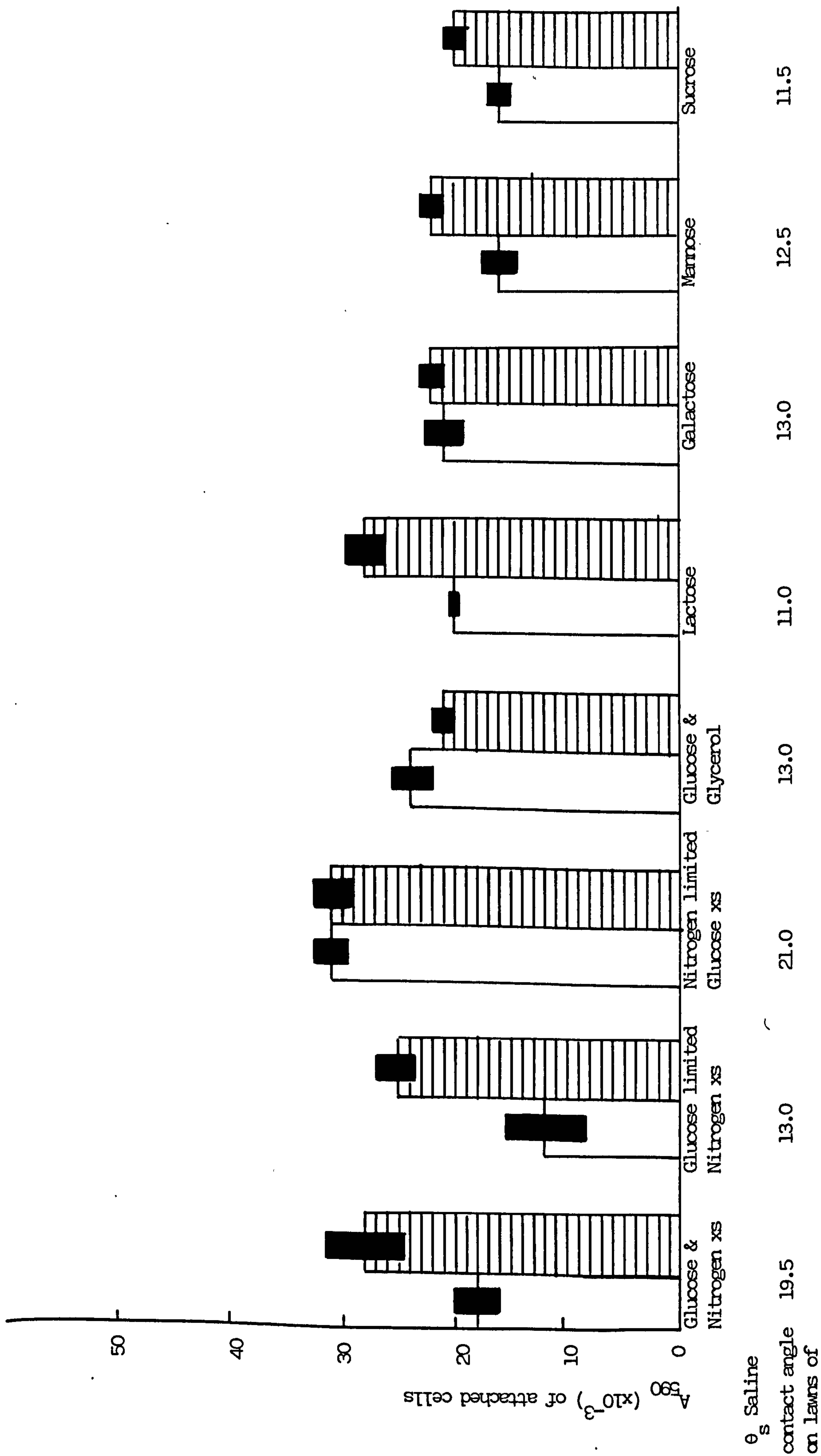


Figure 4.6 The effect of carbon/nitrogen ratio and carbon source on the cell surface characteristics (measured by contact angles) and attachment of the *Flexibacter* sp (60 minutes attachment)

(□), cells attached to PD surface; (■), cells attached to TCD surface; (▨), 95% confidence limits of the mean (n = 8)

medium there was only a slight difference in attachment between the two surfaces (Fig. 4.3). The Chromobacterium sp, after 24 hrs growth attached in approximately equal numbers to both surfaces after growth in glucose-limited medium; however, after growth in sucrose, attachment to the PD surface was far higher than to the TCD surface (24 hr growth, Fig. 4.4). Similarly, the Flexibacter sp, showed equal attachment levels to both surfaces after growth in nitrogen limited conditions, though higher attachment to the TCD surface in glucose limited conditions (Fig. 4.6).

Differences in bacterial cell surface characteristics were demonstrated by saline contact angle (θ_s) measurements on lawns of cells after growth in the different media. Variations in the bacterial cells θ_s 's are related to the hydrophobicity of the cell surface; the larger θ the more hydrophobic the cell surfaces; the smaller θ the more hydrophilic the surface (Chapter 5).

The large variations in bacterial cell surface characteristics with culture conditions can be clearly seen from Figures 4.2 - 4.6 (Appendix Tables 6-8), e.g. Pseudomonas fluorescens shows a range of θ_s from 17.5° after growth in glucose and nitrogen sufficient medium to 8.5° when mannose was the carbon source (Fig. 4.2). The largest differences were shown by the Chromobacterium sp. which had θ_s of 75° after 24 hrs growth in galactose and 10.5° after 24 hrs growth in lactose (Fig. 4.4). This bacterium was in general the most hydrophobic. The contact angles for the Chromobacterium sp, not only varied with growth condition, but also between 24 hr and 48 hr growth (Fig. 4.4 and 4.5).

The variations in cell surface characteristics was reflected in the colony form and amount of polymer produced by the bacteria, and was demonstrated by growing the four species on different carbon/nitrogen ratio and carbon source solid media (Table 4.2). Large differences in polymer production alone were caused by growth on the various media,

TABLE 4.2 Polymer production and growth on solid media of different C/N ratios and carbon substrates

(NH ₄ ⁺ N source)					
C source C/N ratio	Pseudomonas fluorescens	Enterobacter cloacae	Chromobacterium sp	Flexibacter sp	
Glucose Nitrogen xs	^a xxx 2-3mm ^b ++	xxx 2-3mm +++	xxx 1-2mm +	xxx	2mm ++
Glucose ltd Nitrogen xs	xxx <1mm + ₋	xxx 2mm +	xxx 1mm + ₋	xxx	1mm +
Nitrogen ltd Glucose xs	xxx <2mm + ₋	xxx 5-6mm +++	xxx 2mm +	xxx	5mm +++
Gluc/Nitrogen xs + Glycerol	xxx 2mm +++	xxx 3mm ++	xxx 1-2mm ++	xxx	4mm +++
Galactose	x <<1mm -	xxx 5mm ++	xxx 1mm +	xxx	4mm +++ <1mm
Lactose	x <<1mm -	xx 1mm +	xxx 2mm ++	xxx	3mm +++
Sucrose	x <<1mm -	xxx 3mm +	xxx 3mm ++	xxx	0.5mm + ₋
Mannose	xx 0.5mm + ₋	xxx 2mm + ₋	xxx 1mm + ₋	xxx	2-4mm +++

^a x poor, xx moderate, xxx good growth

^b + poor, + small, ++ moderate, +++ good polymer production

mm = colony size

these differences were not necessarily related to the extent of bacterial growth. The amount of polymer produced varied not only with growth condition, but also with the species of bacterium, and underlines the extent of the cell surface variability.

A comparison of the attachment shown by these four bacterial species in glucose limited, nitrogen limited and glucose/nitrogen sufficient media after 24 hours growth in batch culture and after growth in continuous culture ($D = 0.025 \text{ h}^{-1}$), indicates a possible further variation in bacterial surface characteristics. The attachment levels are often quite different between batch culture and continuous culture, e.g. P. fluorescens after growth in glucose limited batch culture attached more to the PD surface and less to the TCD surface than after growth in continuous culture of similar medium (Table 4.3). Similarly large variations can be found for each bacterial species under each set of culture conditions (Tables 4.3-4.7; Appendix Table 6-9). Again the degrees of attachment to the two solid surfaces were independently variable with culture condition, i.e. batch and continuous culture, for each bacterial species.

4.4.2 The Effect of Growth Rate on Bacterial Attachment and Cell Surface Characteristics

The effect of dilution rate on bacterial attachment varied with the organism, as did its affect on bacterial cell surface characteristics (Tables 4.7-4.10; Appendix Tables 10-13). Both the initial levels of attachment at 5 mins, to both surfaces and the final levels of attachment, at 60 mins, were affected by bacterial growth rate. The two bacteria which underwent flocculation, i.e. E. cloacae and the Chromobacterium sp, show the largest variation in cell surface characteristics, while the cell surface characteristics of the two non-flocculating species were more stable, i.e. P. fluorescens and the Flexibacter sp, (Tables 4.7-4.10).

These two categories of flocculating and non-flocculating bacteria can be further subdivided, since one member of each category attached well, while the other was relatively poor in its attachment to the solid surfaces. P. fluorescens and E. cloacae (Tables 4.7-4.8) showed relatively poor attachment to both surfaces at all dilution rates. However,

TABLE 4.3 To compare the attachment of Pseudomonas fluorescens to polystyrene surfaces after growth in a chemostat (D = 0.025 h⁻¹) and batch culture in different carbon/nitrogen ratio media

Culture Condition	(60 mins attachment)					
	Glucose Limited A ₅₉₀ (x10 ⁻³) attchd cells		Nitrogen Limited A ₅₉₀ (x10 ⁻³) attchd cells		Glucose/Nitrogen xs A ₅₉₀ (x10 ⁻³) attchd cells	
	PD	TCD	PD	TCD	PD	TCD
Chemostat Culture (D=0.02h ⁻¹)	14 ([±] 3) ^a	81 ([±] 3)	9 ([±] 3)	85 ([±] 11)	6 ([±] 2)	17 ([±] 2)
Batch Culture	27 ([±] 3)	39 ([±] 5)	40 ([±] 2)	36 ([±] 4)	24 ([±] 3)	38 ([±] 3)

^aParentetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.4 To compare the attachment of Enterobacter cloacae to polystyrene surfaces after growth in a chemostat (D = 0.025 h⁻¹) and batch culture in different carbon/nitrogen ratio media

Culture Condition	(60 min attachment)					
	Glucose Limited A ₅₉₀ (x10 ⁻³) attchd cells		Nitrogen Limited A ₅₉₀ (x10 ⁻³) attchd cells		Glucose/Nitrogen xs A ₅₉₀ (x10 ⁻³) attached cells	
	PD	TCD	PD	TCD	PD	TCD
Chemostat Culture (D=0.025h ⁻¹)	4 ([±] 2) ^a	24 ([±] 3)	26 ([±] 2)	37 ([±] 5)	10 ([±] 2)	28 ([±] 2)
Batch Culture	10 ([±] 4)	32 ([±] 3)	13 ([±] 3)	19 ([±] 0.8)	11 ([±] 3)	24 ([±] 3)

^aParentetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.5 To compare the attachment of the Chromobacterium sp, to polystyrene surfaces after growth in a chemostat ($D = 0.025h^{-1}$) and batch culture in different carbon/nitrogen ratio media

Culture Condition	(60 mins attachment)					
	Glucose Limited $A_{590}(x10^{-3})$ attchd cells		Nitrogen Limited $A_{590}(x10^{-3})$ attchd cells		Glucose/Nitrogen xs $A_{590}(x10^{-3})$ attched cells	
	PD	TCD	PD	TCD	PD	TCD
Chemostat Culture ($D=0.025h^{-1}$)	17 (± 5) ^a	50 (± 3)	18 (± 3)	28 (± 4)	32 (± 3)	50 (± 5)
Batch Culture	99 (± 9)	108 (± 3)	70 (± 5)	63 (± 2)	59 (± 10)	70 (± 3)

^aParenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.6 To compare the attachment of the Flexibacter sp, to polystyrene surfaces after growth in a chemostat ($D = 0.025 h^{-1}$) and batch culture in different carbon/nitrogen ratio media

Culture Condition	(60 min attachment)					
	Glucose Limited $A_{590}(x10^{-3})$ attchd cells		Nitrogen Limited $A_{590}(x10^{-3})$ attchd cells		Glucose/Nitrogen xs $A_{590}(x10^{-3})$ attchd cells	
	PD	TCD	PD	TCD	PD	TCD
Chemostat Culture ($D=0.025h^{-1}$)	17 (± 3) ^a	31 (± 2)	3 (± 2)	17 (± 2)	9 (± 0.8)	16 (± 2)
Batch Culture	12 (± 7)	25 (± 3)	31 (± 3)	31 (± 3)	18 (± 4)	28 (± 7)

^aParenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.7 The effect of growth rate on cell surface characteristics
measured by Interaction Chromatography and attachment of
Pseudomonas fluorescens

Chemostat Dilution Rate (h ⁻¹)	Interaction Chromatography (% counts retained in columns)			A ₅₉₀ (x10 ⁻³) cells attached to PD		A ₅₉₀ (x10 ⁻³) cells attached to TCD	
	Hydrophobic Chromato- graphy column	Anionic Exchange Resin	Cationic Exchange Resin	5 min	60 min	5 min	60 min
0.05	16.1	16.5	83.0	12 (⁺ 2) ^a	25 (⁺ 3)	21 (⁺ 3)	27 (⁺ 3)
0.1	17.9	10.2	85.9	6 (⁺ 3)	30 (⁺ 7)	14 (⁺ 0.8)	25 (⁺ 3)
0.15	12.0	2.3	77.5	9 (⁺ 0.8)	20 (⁺ 2)	19 (⁺ 2)	32 (⁺ 3)
0.2	14.6	8.9	84.1	6 (⁺ 2)	32 (⁺ 4)	13 (⁺ 2)	21 (⁺ 3)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.8 The effect of growth rate on cell surface characteristics
measured by Interaction Chromatography and attachment of
Enterobacter cloacae

Chemostat Dilution Rate (h ⁻¹)	Interaction Chromatography (% counts retained in columns)			A ₅₉₀ (x10 ⁻³) cells attached to PD		A ₅₉₀ (x10 ⁻³) cells attached to TCD	
	Hydrophobic Chromato- graphy column	Anionic Exchange Resin	Cationic Exchange Resin	5 min	60 min	5 min	60 min
0.05	45.0	33.1	80.1	8 (⁺ 2) ^a	31 (⁺ 3)	19 (⁺ 3)	43 (⁺ 4)
0.1	79.9	79.0	92.9	7 (⁺ 0.8)	18 (⁺ 2)	17 (⁺ 0.8)	31 (⁺ 0.5)
0.15	86.2	1.8	62.8	3 (⁺ 3)	5 (⁺ 3)	17 (⁺ 3)	24 (⁺ 3)
0.2	6.4	1.8	54.4	5 (⁺ 0.8)	8 (⁺ 0.5)	17 (⁺ 2)	22 (⁺ 4)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.9 The effect of growth rate on cell surface characteristics measured by Interaction Chromatography and attachment of the Chromobacterium sp.

Chemostat Dilution Rate (h ⁻¹)	Interaction Chromatography (% counts retained in columns)			A ₅₉₀ (x10 ⁻³) cells attached to PD		A ₅₉₀ (x10 ⁻³) cells attached to TCD	
	Hydrophobic Chromatography column	Anionic Exchange Resin	Cationic Exchange Resin	5 min	60 min	5 min	60 min
0.05	10.0	32.3	6.7	14 (⁺ 3) ^a	34 (⁺ 6)	16 (⁺ 3)	13 (⁺ 4)
0.1	32.4	24.0	21.6	14 (⁺ 0.8)	37 (⁺ 2)	25 (⁺ 2)	39 (⁺ 3)
0.15	61.4	54.7	94.9	21 (⁺ 4)	70 (⁺ 3)	23 (⁺ 4)	59 (⁺ 5)
0.2	30.0	44.4	94.1	27 (⁺ 3)	84 (⁺ 8)	29 (⁺ 0.8)	56 (⁺ 4)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 4.10 The effect of growth rate on cell surface characteristics measured by Interaction Chromatography and attachment of the Flexibacter sp.

Chemostat Dilution Rate (h ⁻¹)	Interaction Chromatography (% counts retained in columns)			A ₅₉₀ (x10 ⁻³) cells attached to PD		A ₅₉₀ (x10 ⁻³) cells attached to TCD	
	Hydrophobic Chromatography column	Anionic Exchange Resin	Cationic Exchange Resin	5 min	60 min	5 min	60 min
0.05	73.1	87.6	62.4	9 (⁺ 2)	15 (⁺ 3)	7 (⁺ 3)	7 (⁺ 3)
0.1	60.2	91.5	87.0	13 (⁺ 2)	51 (⁺ 5)	13 (⁺ 2)	76 (⁺ 7)
0.15	44.2	99.9	100	10 (⁺ 0.8)	101 (⁺ 17)	17 (⁺ 3)	90 (⁺ 15)
0.2	55.7	62.7	73.5	32 (⁺ 3)	108 (⁺ 23)	25 (⁺ 3)	111 (⁺ 10)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

fluctuations in their attachment were associated with changes in cell surface characteristics. The Chromobacterium sp, and the Flexibacter sp, both showed increased attachment to both surfaces with increases in dilution rate. Not only did their levels of attachment vary with dilution rate, but so did their cell surface characteristics (Table 4.9-4.10).

The changes in growth rate occasionally also caused the attachment to PD and TCD surfaces to alter their proportions. The Chromobacterium sp, showed consistently higher attachment levels to PD than TCD at all dilution rates except 0.2 h^{-1} when there was no apparent difference between the two surfaces (Fig. 4.9). The Flexibacter sp showed higher attachment to TCD than PD at the two lower dilution rates, but the reverse at the dilution rates of 0.15 and 0.2 h^{-1} (Fig. 4.10).

Attachment of the Flexibacter sp, to both solid surfaces was consistently greater at the two higher dilution rates in all the chemostat experiments (Table 4.10, Fig. 4.10; Appendix Table 13), whereas the other species showed no such consistent relationship. Comparison of a time series of attachments of the 4 bacterial species and the 4 dilution rates (Fig. 4.7-4.10) underlines differences between the Flexibacter sp, and the other bacterial species.

P. fluorescens and E. cloacae showed no large changes in adhesion on increased contact time with the substratum at any dilution rate, thus the 5 min levels of attachment to both surfaces may not be different from the 60 min level of adhesion, e.g. attachment of P. fluorescens after growth at dilution rate 0.1 h^{-1} and E. cloacae after growth at 0.2 h^{-1} (Fig. 4.7 and 4.8). The Chromobacterium sp, (Fig. 4.9) increased attachment at the 3 lower dilution rates between 5 and 60 mins, though the extent varied with the substratum, attachment to the TCD surface rose more than that to the PD surface. However, at $D = 0.2 \text{ h}^{-1}$ the Chromobacterium sp, attached less than at any other growth rate. At $D = 0.2 \text{ h}^{-1}$ the Chromobacterium sp's attachment to the two surfaces was approximately equal and there was no

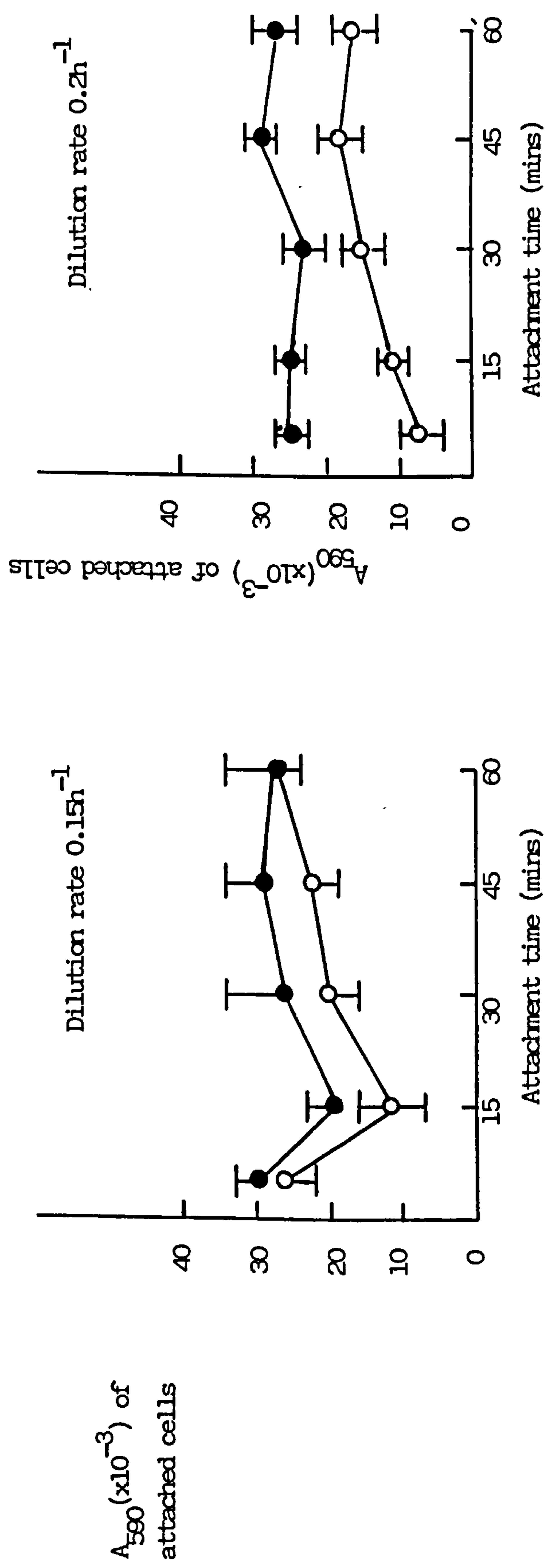
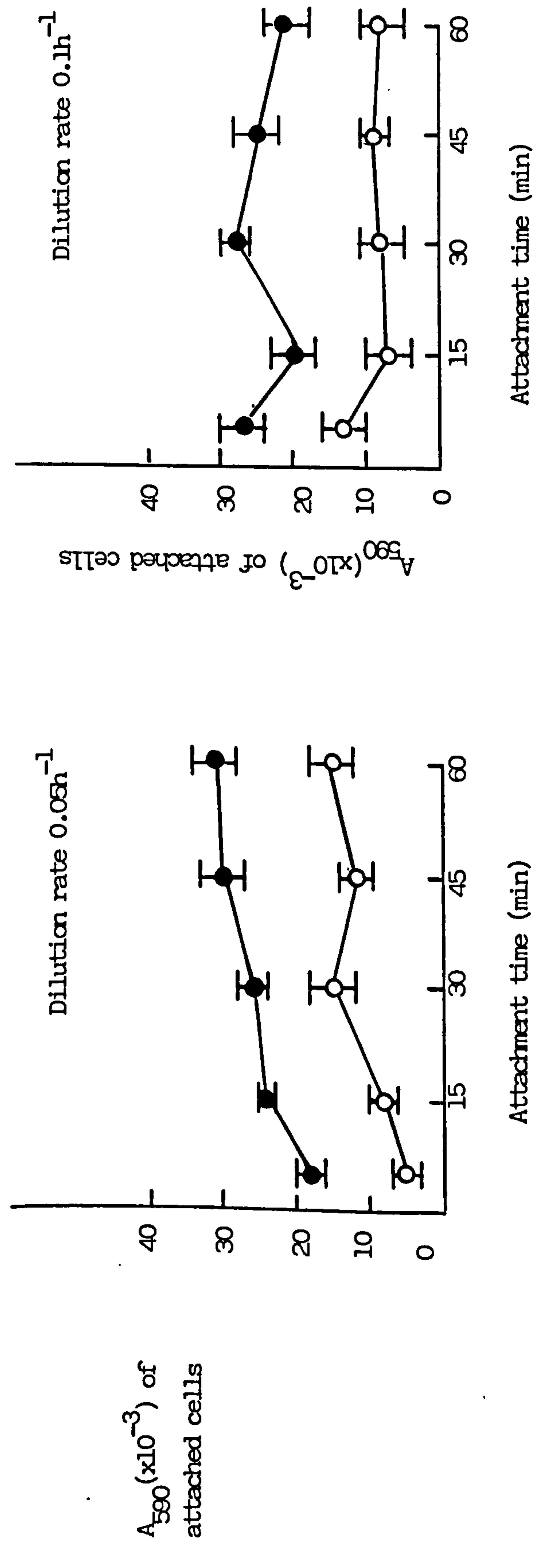


Figure 4.7 The effect of growth rate in chemostat culture on the attachment of Pseudomonas fluorescens to polystyrene surfaces.

(O), cells attached to PD surface; (●), cells attached to TCD surface; The bars represent the 95% confidence limits of the mean (n = 8)

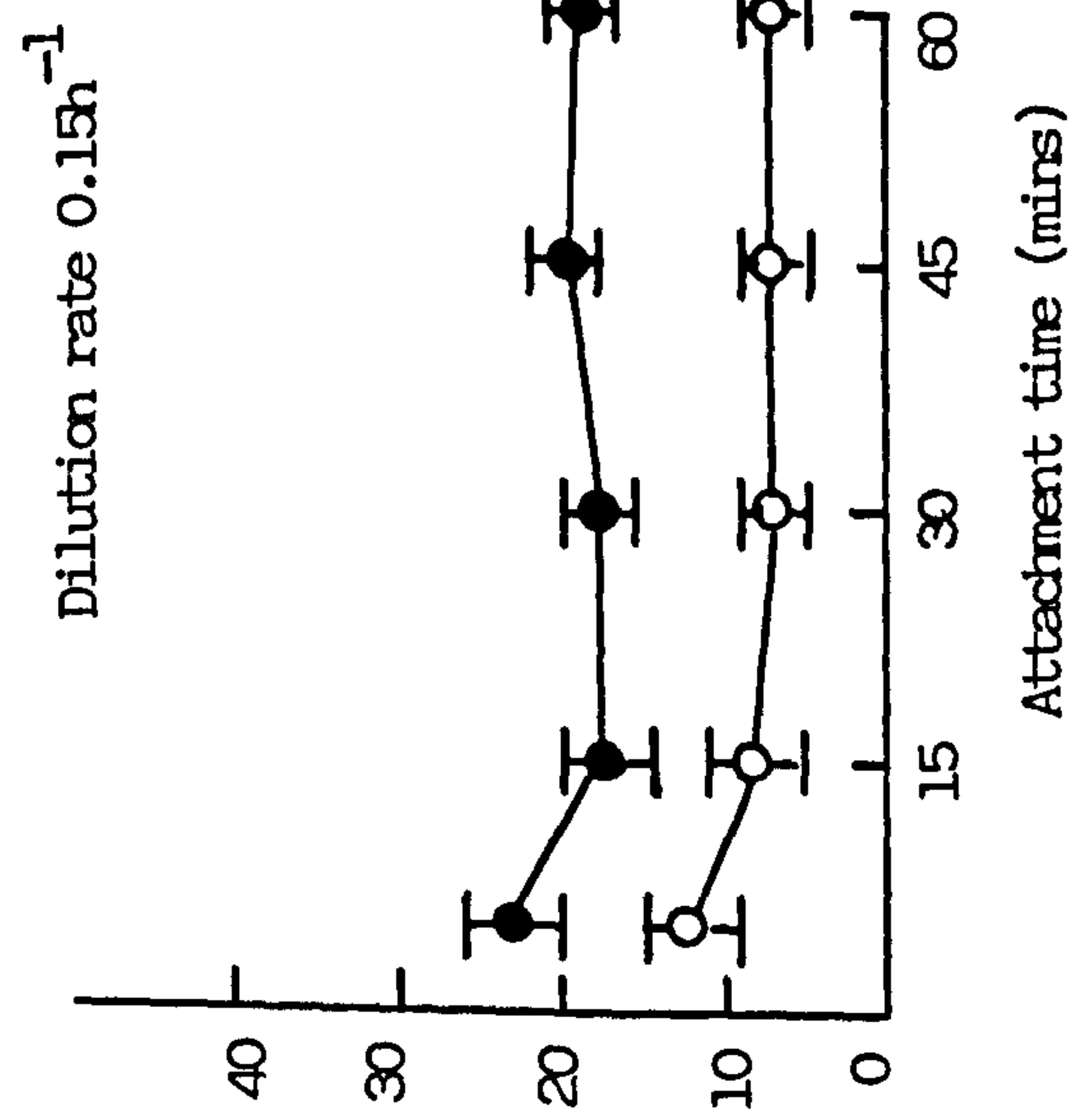
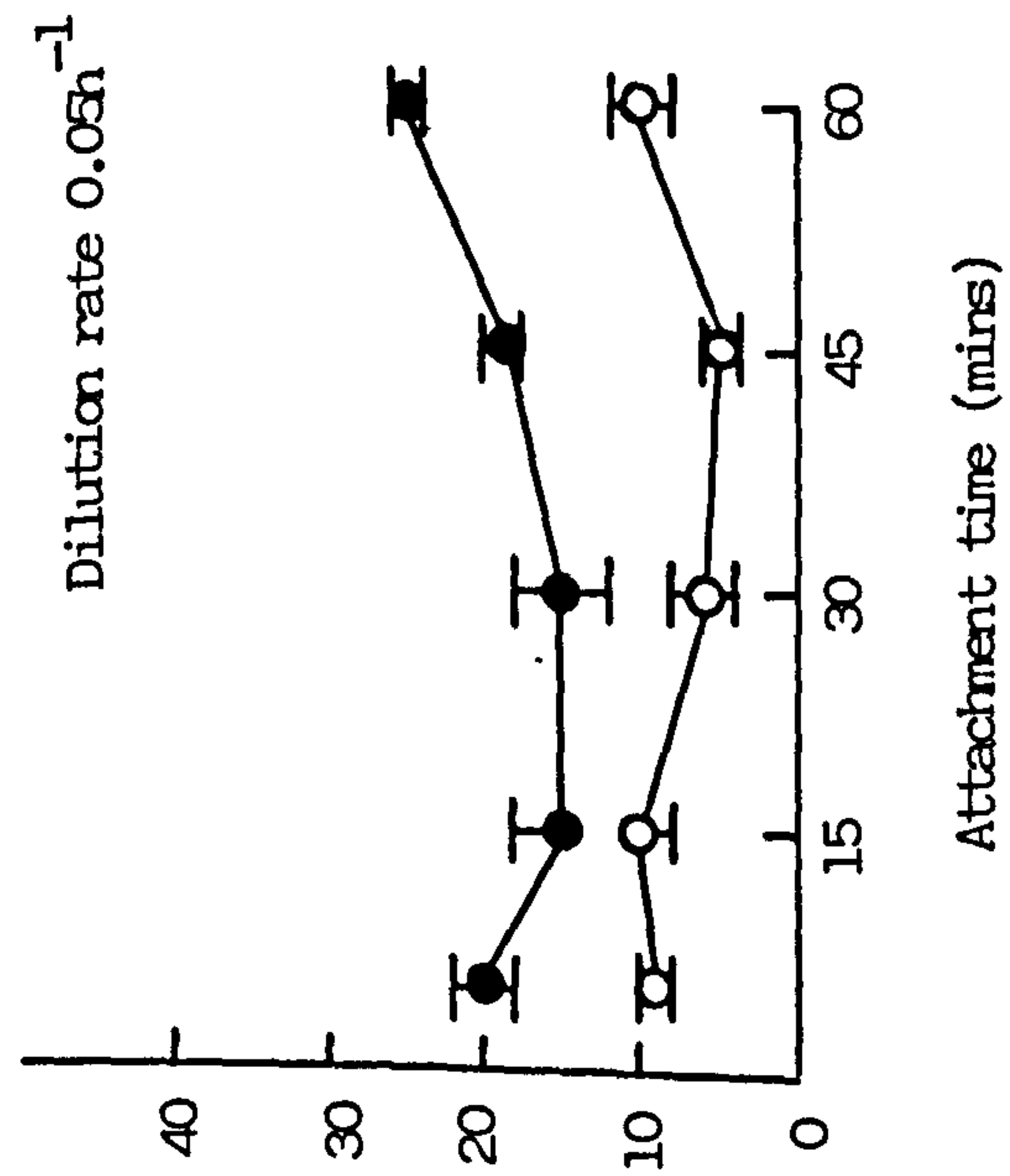
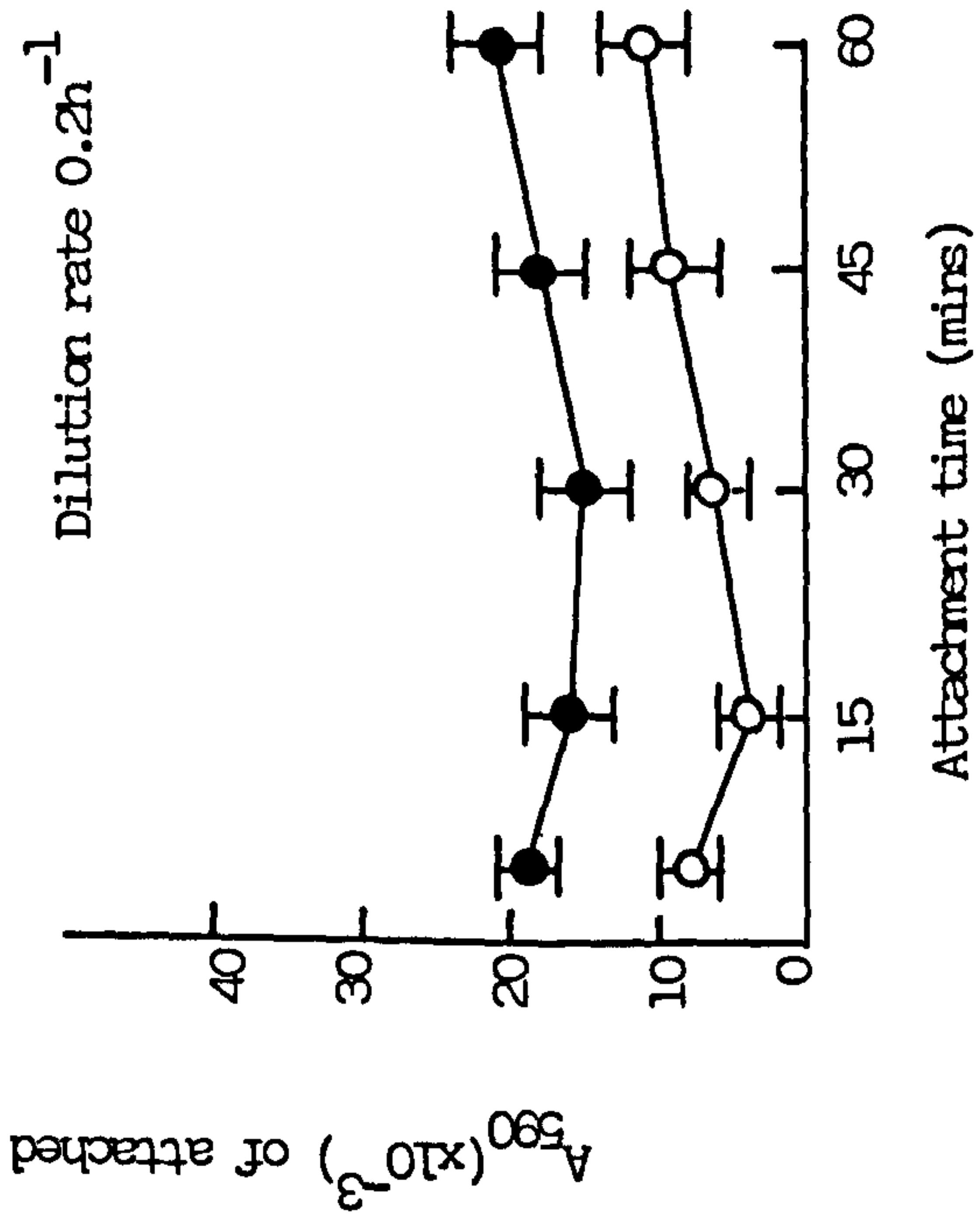
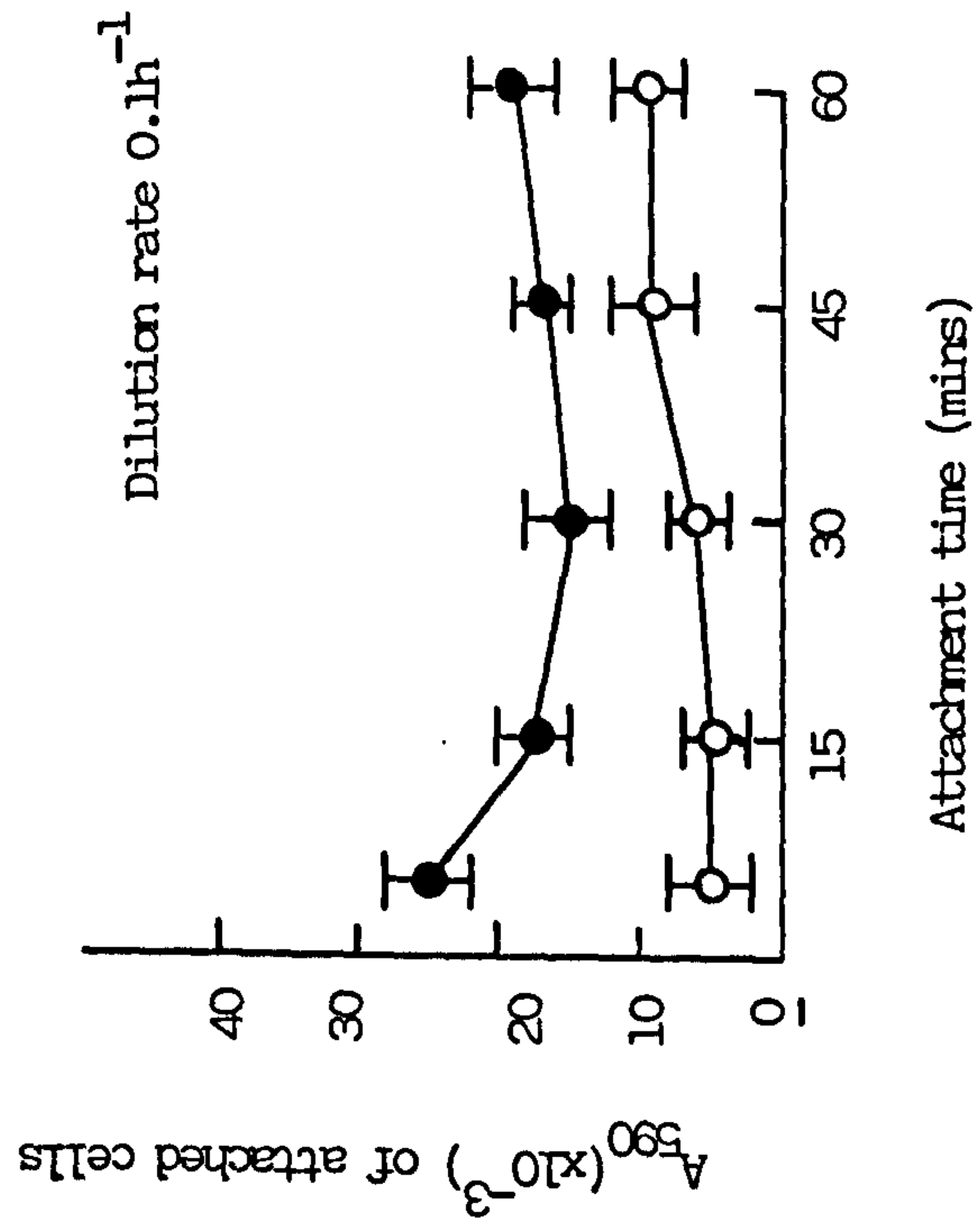


Figure 4.8 The effect of growth rate in chemostat culture on the attachment of Enterobacter cloacae to polystyrene surfaces (○), cells attached to PD surface; (●), cells attached to TCD surface; The bars represents the 95% confidence limits of the mean (n = 8)

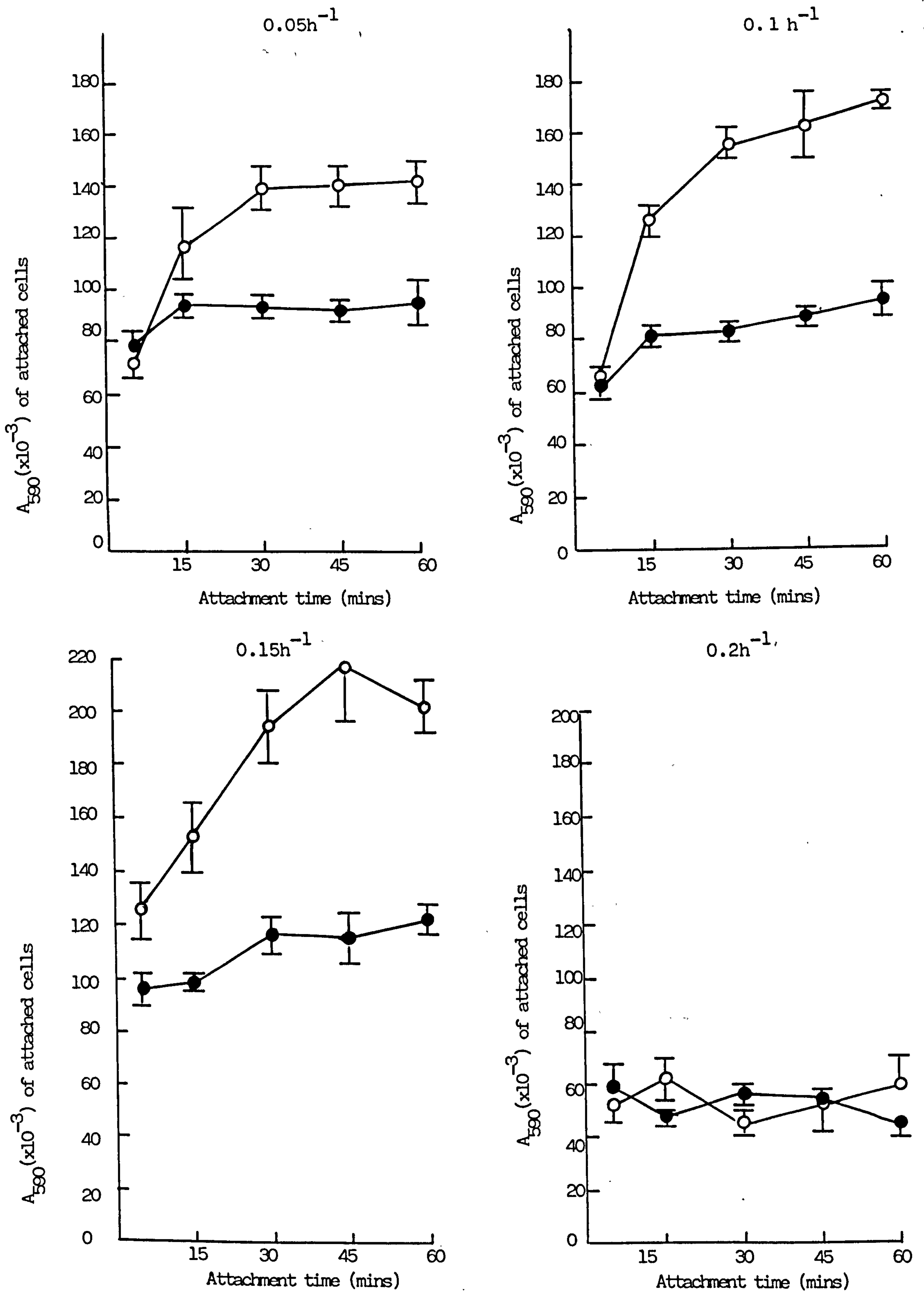


Figure 4.9 The effect of growth rate in chemostat culture on the attachment of the Chromobacterium sp to polystyrene surfaces

(○), cells attached to PD surface; (●), cells attached to TCD surface;
The bars represent the 95% confidence limits of the mean (n = 8)

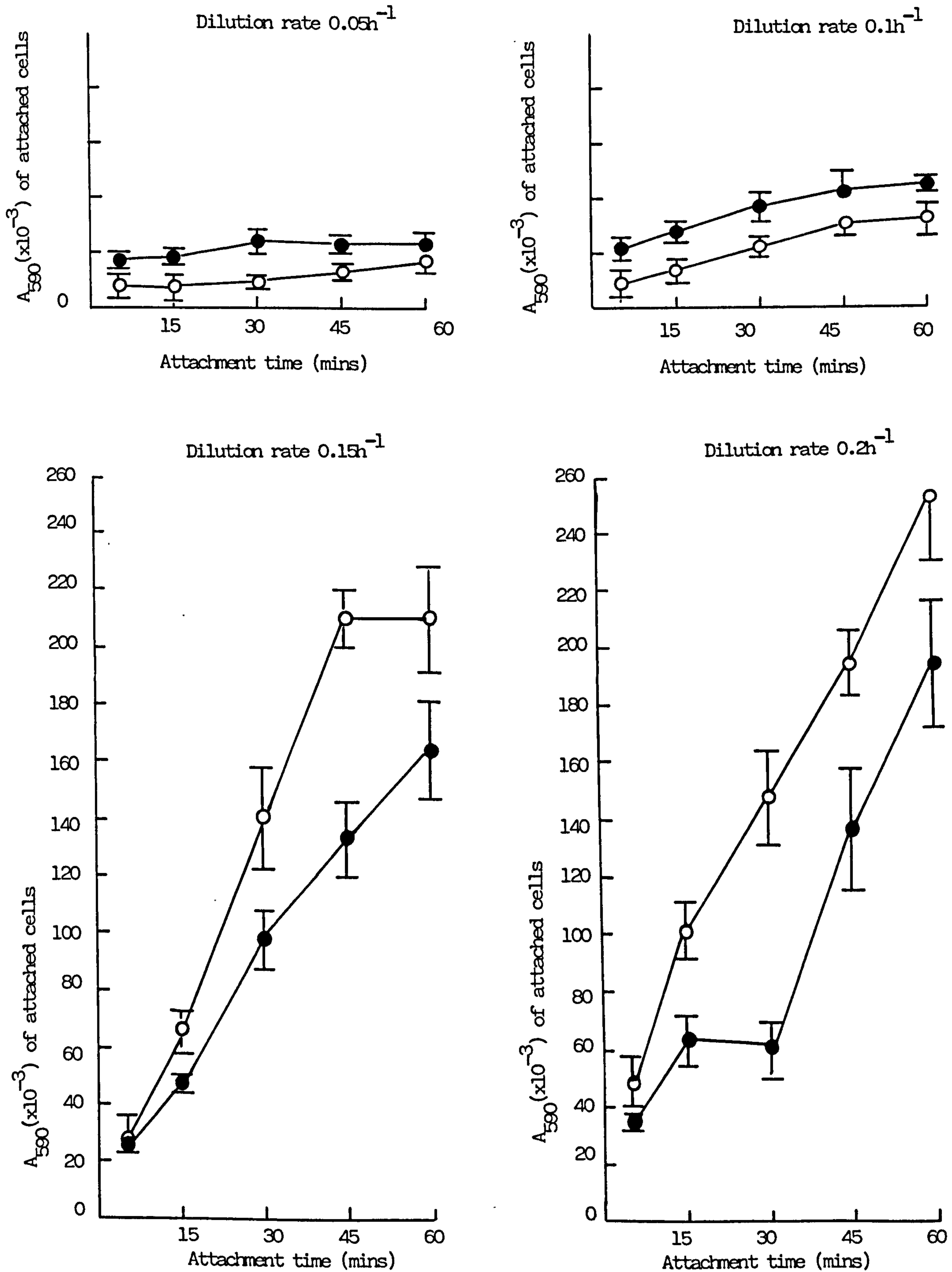


Figure 4.10 The effect of growth rate in chemostat culture on the attachment of the Flexibacter sp to polystyrene surfaces

(O), cells attached to PD surface; (●), cells attached to TCD surface;

The bars represent the 95% confidence limits of the mean (n = 8)

increase in levels of attachment with time (Fig. 4.9).

The Flexibacter sp, however reacted differently from any of the other species. This gliding bacterium increased levels of attachment with time except at the lowest dilution rate, where there was no difference between 5 and 60 mins. At all the remaining dilution rates there was a progressive increase in attachment with time and the rate of increase at growth rates of 0.15 and 0.2 h⁻¹ was considerable (Fig. 4.10).

Thus the relationship between attachment and growth rate for three of the species, P. fluorescens, E. cloacae, the Chromobacterium sp, seems largely based on changes of bacterial cell surface characteristics. The attachment of the Flexibacter sp, after growth at different dilution rates may not only be the result of changes in cell surface characteristics but may involve other components, that of bacterial activity. The Flexibacter sp's attachment increased with dilution rate in continuous culture as would its physiological activity.

The results shown by the continuous culture experiments and the batch culture experiments were occasionally slightly at variance with similar and repeat experiments (Tables 4.3-4.9; Figs. 4.1-4.10 and Appendix Tables 6-13 (repeat experiments)). Thus although both the growth and experimental conditions were controlled there were still differences in attachment results. This illustrates how sensitive attachment properties may be to changes in environmental variables not under control or defined.

4.5 DISCUSSION

4.5.1 The Variability of the Bacterial Cell Surface and its Effects on Bacterial Attachment to Solid Surfaces

The results presented in this Chapter clearly indicate a wide variation of bacterial attachment levels, for all the species investigated, with bacterial culture conditions, e.g. the carbon source, and with growth

rate. These variations were apparently because of phenotypic changes related to differences in bacterial cell surface characteristics measured by changes in θ_s , HIC and EIC interactions. However, each species behaves differently and thus the basis of the changes are the genotypic components which determine individual species.

The growth medium of bacteria has previously been shown to be important in subsequent bacterial attachment in a range of environments. Daniels (1980) reviewed data from Peele indicating that bacteria grown on slightly acidic media attached to a greater extent to soil particles than those bacteria cultivated on alkaline media. He also indicated from data presented by Adamov, that the moisture content of agar affected bacterial adsorption by tri CaPO_4 , since bacteria grown on dried agar adsorbed more heavily than those from moist agar (Daniels, 1980). Although no explanation was offered for the effect, Jones et al. (1976) found that the growth medium changed the attachment of Vibrio cholerae to rabbit epithelium. After growth in trypticase soy broth there was significant attachment, but after growth on trypticase soy agar there was little adhesion by V. cholerae to the epithelium. They found that the effect was not related to cell motility. Similarly, cultures of a strain of Bacteroides prodigiosum grown on meat peptone agar (MPA), Ashby's medium, bean agar and cabbage agar adsorbed equally, but after growth in meat peptone broth (MPB) attachment decreased. While a Scarcina sp, grown on MBP hardly underwent any attachment to glass surfaces, growth on MPA promoted strong adsorption (Zvyagintsev, 1959).

Baier (1980b) demonstrated that the water contact angle of confluent bacterial cultures grown on a series of different agars varied with the type of agar and the bacterial species, e.g. Staphylococcus albus on trypticase soy agar had a water contact angle (θ_w) of 0° whereas on blood agar θ_w was 12° . Although these changes were not related to the subsequent

attachment of the bacteria, the results described by the various workers above indicate a possible relationship between the growth conditions, attachment and the nature of the bacterial surface.

Further indications that bacterial cell surface chemical and physical properties are of importance in bacterial attachment have been shown indirectly by several workers. Heat and formalin killed bacteria have their macromolecular cell wall structure irreversibly altered, whereas the cell wall of those killed by ultra-violet (UV) light should not have received structural damage. Aeromonas liquifaciens N.C.T.C. 8196, and Pseudomonas fluorescens N.C.I.B. 9046 treated with heat or formalin were found to considerably reduce their attachment to solid surfaces compared to untreated cells. UV killed cells only showed slightly lower levels of attachment than the control cells (Meadows, 1971). Corpe, (1973) working with marine bacteria also found heat and formaldehyde killed bacteria did not attach to surfaces. Formaldehyde treatment of a marine pseudomonad and of other marine bacteria inhibited their subsequent attachment to solid surfaces (Fletcher, 1980b). However, the effect is not always inhibitory and formaldehyde treatment has been shown to increase the attachment of marine bacteria Corynebacterium erythrogenes, a Micrococcus sp, and Bacillus filicolicus (J.J. Bright, cited in Fletcher, 1980b), and leave unaffected the attachment of a Streptococcus sp, to glass. Heat treated and formalin treated cells of Staphylococcus epidermidis and Staphylococcus saprophyticus also did not change their attachment to FEP-Teflon nor to cellulose acetate (Hogt et al. 1982). The reaction of UV killed bacteria also varies. Cells of a UV killed Streptococcus sp, attached normally to surfaces (Orstavik, 1977), while UV treatment was found to reduce the attachment of a marine Pseudomonas sp, (J.J. Bright, cited in Fletcher, 1980b).

These variations in the effect of heat and formalin treatments with

species may find their basis in the original macromolecular structure at the bacterial cell surface, and the manner in which this structure is changed by the treatment both chemically and structurally. The alterations in structure might result in a macromolecular arrangement favouring bacterial attachment, impairing bacterial attachment or causing no real change in the likelihood of bacterial adhesion occurring.

Baier (1980) indicated that the cell walls of Bacillus megaterium dried both at 5°C and 80°C on platinum from a water suspension had a contact angle with water of 26°, while E. coli dried at 5°C and 80°C had a contact angle of 35° and 58° respectively. He attributed this difference between Gram-positive and Gram-negative organisms to the difference in the cell wall structures, particularly the presence of lipid in the outer lipopolysaccharide layer of E. coli. This shows the effect of heat treatment on the cell walls of bacteria, and indicates another explanation why the heat treated Gram-positive Bacillus megaterium (Baier 1980) varied little in their attachment from untreated cells.

The influence of UV may vary with bacterium depending on the energy requirements of maintaining the cell wall structures. Although UV treatment does not directly modify the macromolecular components of cell walls it may do so indirectly since the death of a cell may result in changes in the constituents at the cell surface through lack of maintenance. The susceptibility of bacterial cell walls to rapid change because of this may vary genotypically and may also be influenced by the bacterium's previous metabolic state.

Thus changing bacterial cell surface characteristics have been demonstrated to be a major influence on bacterial attachment to solid surfaces. Both artificial modifications to cell surface characteristics and phenotypic differences caused by varying nutrient conditions (the results presented in this Section), cause changes in the physico-chemical

interactions between the bacterial surface and the solid surface (Chapter 5). Not only do cell surface characteristics vary with growth conditions (Figs. 4.2-4.6) but also with bacterial growth rate (Table 4.7-4.10). Such effects vary with species and cause different attachment levels to the two surfaces.

4.5.2 The Influence of Growth Conditions on Bacterial Cell Wall Constituents and Exopolymer Production

Since the bacterial cell surface apparently has a large influence on bacterial attachment its nature and propensity to change is worthy of consideration here. The Gram-negative bacterial cell surface consists of an array of different macromolecules, structural proteins, enzymes, lipopolysaccharides, extracellular polymers and capsules. The extracellular polymers may be homopolymers, e.g. glucans, but are more normally heteropolymers consisting of, among other things, uronic acids and/or pyruval ketal groups (Chapter 1). Variability in these constituents macromolecules will cause variations in the chemical and physical nature of the surface presented to the external environment and, therefore, involved in bacterial attachment..

Both the dilution rate and the limiting nutrient in continuous culture systems have been shown to affect bacterial cell wall constituents. Indeed, the whole elemental composition, i.e. carbon, hydrogen and nitrogen, with the exception of oxygen has been found to change with specific growth rate in continuous culture over a range from 0.035 to 0.71 h⁻¹ for Klebsiella pneumoniae. The molecular weight of the cellular components of this organism rose with growth rate (Esener, 1982). The bacterial cell wall of Aerobacter aerogenes in both Mg²⁺-limited and carbon-limited chemostats showed a decrease in mass per total weight while increasing growth rate from 0.05 to 0.7 h⁻¹ (Tempest & Ellwood, 1969; cited in 1972). However, this effect varies with species and the cell wall of Staphylococcus

aereus showed only slight changes with dilution rate in Mg^{2+} and PO_4^{3-} limited chemostats (Tempest & Ellwood, 1969; cited in 1972).

Variations in the cell wall constituents of Aerobacter aerogenes with growth rate and growth limiting substrate were found to be considerable. Glycerol limited cultures showed an increase in KDO and heptose contents with dilution rate and an increase in cell wall carbohydrate, indicating an increase in the lipopolysaccharide content of the wall. There was also a variation in protein constituents which lowered with increasing dilution rate. A magnesium (Mg^{2+})-limited culture of A. aerogenes showed only slight increases in carbohydrate content of the cell wall with increasing growth rate, while KDO and heptose contents actually decreased. The protein in the wall again fell slightly with increasing growth rate. The phosphorous content varied markedly. Phosphate (PO_4^{3-})-limited cultures, however, showed the least significant changes in cell wall constituents with changes in growth rate. A comparison of the levels of KDO, heptoses, carbohydrates, proteins and phosphorous between the three growth-limiting substrates shows that the levels of each was different for each limiting condition (Tempest & Ellwood, 1969; cited in 1972).

Thus major differences in the cell wall constituents of a bacterium occur with different limiting conditions and at different growth rates. It is such changes that may be reflected in the results presented in this Chapter. The HIC and EIC results over the range of dilution rates 0.05 h^{-1} to 0.2 h^{-1} indicated large variations in the physico-chemistry of the cell surface. These changes were different for each organism and influenced their subsequent attachment to solid surfaces.

It has often been found that the production of exopolymers and the composition of heteropolymers may be affected by the carbon/nitrogen ratio and the carbon source. In the classic work of Dugoid and Wilkinson (1953), the polysaccharide polymer production of Aerobacter aerogenes (type 54) was

investigated in a range of culture conditions. It was found that if carbon was the limiting nutrient then the minimum amount of polysaccharide was produced, while production was maximal if nitrogen, sulphur or phosphate were limiting. Potassium-deficiency resulted in only slight polymer production and Dugoid and Wilkinson suggested that potassium may have been essential for polysaccharide formation. Similar types of results have been indicated by other workers. A Pseudomonas sp, produced no detectable polysaccharide under glucose-limitation, whilst under nitrogen-limitation 43% of the glucose substrate was converted to exopolysaccharide (Williams & Wimpenny, 1978). Corpe (1964) found a carbon/nitrogen ratio of 10:1 was the best growth medium for maximal polysaccharide production for Chromobacterium violaceum. Maximum production of exopolysaccharide by Pseudomonas N.C.I.B. 11264 was found to be in nitrogen limited conditions with excess phosphate and glucose (Williams & Wimpenny, 1980).

However, there have been exceptions to the effect of nitrogen-limitation and carbon-sufficiency inducing polymer production. The formation of alginic acid by Pseudomonas aeruginosa under carbon-limited conditions was substantial (Mion et al. 1978). Another pseudomonad isolated from the River Tay was found to produce polymeric fibrils in both carbon and nitrogen-limited conditions (Ellwood et al. 1982). Still another pseudomonad, Pseudomonas atlantica, increased polymer production with decreasing carbon/nitrogen ratio (Uhlinger & White, 1983). Alginic acid synthesis by Azotobacter vinelandii was high in nitrogen, MoO_4^{2-} , PO_4^{3-} , Fe^{2+} , SO_4^{2-} , Ca^{2+} and K^+ -limiting conditions and remained high even in carbon-limited conditions (Jarmin et al. 1978).

Thus the effect of a limiting nutrient on the amount of polymer production varies with species. The four bacterial species investigated in this Chapter all show depressed levels of polymer production in glucose-limited conditions (Table 4.2) when grown on solid medium. This may

also be partly reflected in the different contact angle measurements found for the cells grown in different carbon/nitrogen liquid media, though exopolymer will not be the only cell wall molecular constituent contributing to θ_s measurements.

Homopolysaccharides require specific carbon sources for their formation due to the specificity of synthesizing enzymes. Streptococcus mutants grow well in the carbon sources of glucose, maltose or fructose, but sucrose is required for the production of the polymer glucan which is synthesized by glucosyl transferases. Heteropolysaccharides, however, are synthesized in varying degrees from any utilizable carbon source, though the extent of production and the constituent molecules might alter with substrate. Growth of E. coli in a high acetate to glucose medium was shown to increase lipid production and depress polysaccharide production, however a lowering of the ratio resulted in the reverse (Dagley & Johnson, cited in Wilkinson, 1958).

The composition of a heteropolymer and the quantity of polymer formed may vary with growth condition; however, the literature also presents evidence to the contrary, suggesting this again is a species dependent factor. The polysaccharides produced by C. violaceum increased when an amino acid was substituted for ammonium as the nitrogen source (Corpe, 1964). The composition of a polysaccharide synthesized by a Pseudomonas sp, did not vary with the type of nutrient-limitation (Williams & Wimpenny, 1978). Nor did the composition of another exopolysaccharide produced by Pseudomonas N.C.I.B. 11264 vary with the carbon substrate, although the quantity produced did change (Williams & Wimpenny, 1977). However, a polysaccharide produced by 'Xanthomonas jleglandis' under different growth-limiting conditions contained different quantities of rhamnose (Ellwood et al. 1978, cited in Pace & Righelato, 1980).

The growth of P. fluorescens, E. cloacae, a Chromobacterium sp, and a

Flexibacter sp, on different carbon sources on solid media shown in this Chapter illustrates the variability in the amount of polymer produced. The variation in θ_s , after growth in liquid culture may partially indicate changes in the extent of polymer production by the bacteria and/or may be partly due to changes in polymer structure. However, any changes in the bacterial envelope may also contribute to θ_s variations. Not only can the molecular composition of the exopolymer vary with nutrient conditions, but also its physical relationship with the bacterial cell wall can change. Corpe (1964) found gelatinous strains of C. violaceum did not produce a polysaccharide matrix, but rather elaborated polysaccharide into the medium when grown in the presence of tryptone. The physical form of the polymer can also be affected by the growth rate, i.e. dilution rate, of bacteria in continuous culture. K. aerogenes produced up to 73% of its polymer at low dilution rates in colloidal form and only 22% in this form at a high dilution rate, with the rest as soluble polymers (Rudd et al. 1982). These differences may reflect changes in the structure of the polymer or a change in the interaction between the polymer and the cell wall.

Further affects of dilution rate in continuous culture on exopoly-saccharide production involve the quantity of exopolymer formed, though again these seem dependent on bacterial species and the polymer produced. Rudd et al. (1983) found that polymer production by K. aerogenes increased as the dilution rate decreased from 0.5 to 0.01 h⁻¹. Similarly, Pseudomonas N.C.I.B. 11264 produced maximal extracellular polysaccharide at growth rates between 0.04 to 0.06 h⁻¹, and decreased as the dilution rate increased over a range 0.023 - 0.235 h⁻¹ (Williams & Wimpenny, 1980). Xanthan gum produced by Xanthamonas campestris increased with decreasing growth rate (0.05 to 0.2 h⁻¹) (Deavin et al. 1977). However, alginic acid synthesis by Azotobacter vinelandii was independent of specific

growth rate over a range of dilution rates 0.05 to 0.25 h⁻¹ (Jarmin et al. 1978), and Pseudomonas aeruginosa produced the same quantity of alginic acid at all growth rates between 0.05 to 0.1 h⁻¹ (Mion et al. 1978).

The effect of growth rate on polymer production then appears to vary with species. In the growth rate experiments described in this Chapter for cultures grown in PYE, it is probably unlikely that changes in cell surface characteristics are dominated by polymer production since the medium was carbon deficient. However, in the chemostat experiments using low growth rates (0.025 h⁻¹) and nitrogen limited conditions, polymer may well have an influence on surface characteristics and the attachment of the bacteria.

Differences in polymer production have been found between batch and continuous culture. Rudd et al. (1982) showed that polymer production was greater in continuous culture than batch culture for K. aerogenes. Indeed, there are major differences between growth phases in batch culture, depending on the bacterial species. The rate of polysaccharide production by K. aerogenes type 54 was greatest in log phase diminishing later in the growth cycle (Dugoid & Wilkinson, 1953). Pseudomonas N.C.I.B. 11264 initiated exopolysaccharide production in late exponential phase and continued to a maximum after the cessation of growth (Williams & Wimpenny, 1977). Not only can the amount of polymer produced be effected by growth phase, but also the composition of the polymer has been found to change. 'P. atlantica' produced an exopolysaccharide which changed composition markedly during the growth cycle in batch culture. Uhlinger and White (1983) showed that the proportion of galactose decreased while uronic acids increased with increased rate of synthesis in the exopolysaccharide of this organism.

Differences in cell surface characteristics with growth phase in batch culture may not be restricted to polymer production, but also to

changes at the cell wall. The electrophoretic mobility of Rhizobium trifolii strain TAl exhibited a sharp increase at late log - early stationary phase while remaining relatively constant over the remaining growth phases (Marshall, 1967). This difference in charge may have been due to cell surface changes, though it is unclear which particular macromolecules were involved.

It has been found that bacteria attach to varying degrees when in different growth phases, dependent on the species. Fletcher (1977) showed a Pseudomonas sp, attached to its highest extent in late log phase, while Zvyagintsev (1973) described three types of attachment with changes in the physiological age of the cells. He found Staphylococcus aureus increased attachment with culture age to a maximum at the initiation of stationary growth phase, Bacteriodes prodigiosum attached most in exponential phase and Bacillus cereus had a sudden and brief peak in attachment in mid-exponential phase.

The four species investigated in this Chapter showed different attachment levels with growth phase in batch culture (Chapter 7). These attachment differences may be related to changes in the cell surface characteristics of these bacteria. This is indicated by the attachments and contact angle measurements of Chromobacterium sp, after growth for 24 hrs and 48 hrs in different carbon source and carbon/nitrogen ratio media (Fig. 4.4 and 4.5). Such changes in the cell surface may be due to differences in polymer production or to changes at the cell wall.

4.5.3 The Effect of Changing Cell Wall Constituents on the Physico-Chemical Interactions of Bacterial Cells, and on Bacterial Attachment

The relationship between the cell wall or polymer constitution and the hydrophobicity or charge interactions of bacterial cells has been substantiated by several workers. A study of the electrophoretic mobility of matt and glossy variants of Streptococcus pyogenes type 6 revealed

changes in charge which were correlated with the accumulation of hyaluronic acid on the bacterial surface during active growth and its removal in the stationary phase (Plummer et al. 1962). The variation in surface structure and electrophoretic mobility, i.e. surface charge for various strains of Rhizobium trifolii was demonstrated by Marshall (1967). Colonial variants differed from the parental form lacking a common polysaccharide type antigen, and they showed reduced surface charge. One variant had a modified type gum production ('mucoid' antigen) and had the largest charge density. There were also differences in charge between a slow-growing and fast-growing Rhizobium sp, which was thought to be associated with the types of surface groups (Marshall, 1967). The influence of genotypic differences on surface type was further demonstrated by Proteus mirabilis strains, a smooth strain, a rough strain and a deep rough strain, which all showed different affinities for hydrocarbons indicating different hydrophobicity of their surfaces (Chapter 5) (Rosenberg et al. 1982).

Thus differences in surface structure are clearly reflected in different charge and hydrophobic characteristics. Not only can phenotypic changes cause these variations, e.g. the results described in this Chapter, but also genotypic differences can be significant. These genotypic changes have been shown to affect attachment. A wild type Pseudomonas fluorescens attached in large numbers whereas a mucoid mutant producing alginate polysaccharide had much lower attachment levels to solid surfaces. A rough strain of the same organism with less polysaccharide in the lipopolysaccharide increased its attachment levels (Pringle, 1983a). Three variants of C. violencium showed different attachment levels to solid surfaces. Two stable non-gelatinous variants which had lost their ability to produce exopolysaccharides also lost their ability to bind to surfaces when compared with a gelatinous variant (Corpe, 1964). Mucoid and non-

mucoid strains of Pseudomonas aeruginosa showed different adherence to hamster tracheal epithelium. The mucoid isolates attached after 4 hrs whereas the non-mucoid form took 6 to 8 hrs to adhere (Baker & Marcus, 1982). However, a non-mucoid variant adhered better to cells of the oropharynx than mucoid cells (Wood et al. 1980). These latter two results, and the data presented in this Chapter, indicate that different bacterial cell surfaces will interact individually with different solid surfaces, i.e. a particular bacterial surface may cause good attachment to one type of solid surface and only poor attachment to another.

The variability of cell surface characteristics and bacterial attachment both with dilution rate and growth conditions in continuous culture, and with batch culture growth conditions demonstrated in this Chapter was considerable. These changes may find their basis in the macromolecular composition of the cell wall and the composition and quantity of the exopolymeric layers. Given the extent of the variations described in the survey of literature in both the cell wall and the polymeric layers caused by different growth conditions and growth rates, it may be advantageous to view the external layers of a bacterial cell as very flexible. Rather than considering the cell wall area as a stable entity it may be considered to be in a state of continual flux. The extent of this flux and the macromolecular composition will vary with species and the physiological state of the cell, which is largely determined by the environmental conditions. Changes at the cell surface will cause differences in the cell surface hydrophobicity, charge and hydrophilicity and in its three-dimensional molecular structure. All these will in turn alter the bacterium's interaction with its environment and its attachment to solid surfaces (For a discussion of the physico-chemical interactions of attachment see Chapter 1 and Chapter 5). Depending on the extent of the potential cell surface variations within a species the range of attachment

levels to solid surfaces will vary.

Genotypic variations also result in different cell surface characteristics and attachment levels. In any natural population of microorganisms there will be a small pool of such variants, and in certain environmental conditions they may be selected for, becoming dominant in the population. In a continuous culture system, Pringle et al (1983a) found the wild type and rough strains of P. fluorescens concentrated on the fermenter walls, but the mucoid form was predominant in the liquid phase.

The particular nature of the physico-chemical changes at the cell surface and subsequent changes in attachment may be caused by several factors. Charged groups such as carboxyl groups, amino groups and phosphate groups may vary their proportions with environmental condition and genotypic type. Differences in nonpolar constituents such as amphiphatic proteins, glycoproteins, and lipids will give the cell changed hydrophobicity. The presence or absence of polymer will cause the masking of cell wall features and the development of new interactions. The extent of the lipopolysaccharide layer will, also, influence the physico-chemistry of the surface.

Not only may the physico-chemistry of a bacterial surface change because of quantitative macromolecular changes, but molecular and steric re-arrangements alone may also affect charge and hydrophobic interactions. Rosenberg et al. (1982) suggested that strains of Proteus mirabilis showing different hydrophobic interactions did so because the phospholipids of the outer and inner leaflets of the outer membrane were in different locations. It was proposed also, that a smooth strain may have had the same number of hydrophobic sites, but that they were shielded from interacting by the long hydrophilic carbohydrate chains of the lipopolysaccharide molecules. Bordetella pertussis has been shown to vary the exposure of proteins at the cell surface or their accessibility although

the same proteins were present, with species and growth conditions (Redhead, 1983).

4.5.4 The Role of Metabolic Activity on Bacterial Attachment to Solid Surfaces

ZoBell (1943) proposed that attachment was not a passive phenomenon but required a period of incubation with nutrients. He suggested that this indicated some synthesis of material may be necessary for attachment to occur. The production of exopolymer has been proposed by several workers to be an important participant in promoting the permanent attachment of bacteria to solid surfaces (Corpe, 1973; Fletcher & Floodgate, 1973; Marshall & Cruickshank, 1973; Marshall et al. 1971a). This suggests an active process which would indicate that (1) attachment would be time-dependent if synthesis of adhesive material were involved; (2) the physiological status of the cell would have a significant influence on attachment.

Increased incubation time has been shown to increase the numbers of attached cells to several different surfaces for a variety of bacterial species. Østavik (1977) showed that the attachment of Streptococcus faecium to glass increased with time, and similarly a marine Pseudomonas sp, increased levels of adhesion with time (Fletcher, 1977). This increase in attachment may partly be due to an increase in the probability of a bacterium coming into contact with the substratum, since increasing culture concentration has a similar effect (Fletcher, 1977; Chapter 7). Thus a physiological process is not the only component contributing to increasing attachment with incubation time.

The results presented in this Chapter investigating the influence of growth rate and attachment time on bacterial adhesion, indicate that for some species, e.g. E. cloacae, there was no change in attached numbers with attachment time (Fig. 4.8), while for other species, e.g. the

Flexibacter sp (Fig. 4.10), there was an increase in bacterial attachment with time. Even within a species, a change in growth rate could cause a difference in the time dependence of attachment, e.g. the Chromobacterium sp, (Fig. 4.9).

Since bacteria generally increase metabolic activity with growth rate, more bacteria might be expected to attach at higher growth rates if attachment involves a physiologically related process. However, if the production of polymer is involved in the firm adhesion of bacteria to surfaces the evidence presented in the literature above suggests that growth rate has a variety of effects on its production depending on species. Sometimes polymer synthesis is maximal at low dilution rates. Moreover, there is evidence suggesting that polymer production by bacteria may actually inhibit attachment (see Chapter 8 for a discussion of the role of polymers in bacterial adhesion). It should be noted from the experiments described in this Chapter that only the Flexibacter sp, consistently increased attachment with growth rate and incubation time. This suggests the possibility that of the four species investigated only the Flexibacter sp, had a direct relationship between attachment and physiological activity.

The attachment of E. cloacae, P. fluorescens and the Chromobacterium sp, then, may not have been active processes, but rather governed by the nature of the cell surface which itself was influenced by bacterial activity. A lack of increase in levels of adsorption with incubation time may have been caused by physico-chemical and steric arrangements of the cell surface not favouring attachment. The Flexibacter sp, however, may possess an active mechanism for attachment to a solid substratum, and it is interesting that this bacterium is capable of a gliding motility across a solid surface. An active process of attachment may be an adaptive phenomenon by this organism (Chapter 7).

The results presented here indicate a major difficulty in generalizing about the mechanisms of bacterial attachment, since each species investigated reacts differently. It may be the case that some bacteria undergo physico-chemical adsorption with no active involvement other than a maintenance of the cell wall status, while still other bacteria may have an active process of attachment, although physico-chemical adsorption will still play a role.

4.5.5 The Effect of Changing Environmental Conditions on Biofilm Development

Differences in growth conditions and growth rates, then, cause changes in bacterial cell surface characteristics. These changes, even apparently small changes, are reflected in large differences in levels of bacterial attachment to solid surfaces. In natural aquatic ecosystems even small fluctuations in environmental parameters might considerably alter bacterial cell surface characteristics and thence their attachment to solid surfaces. This effect would vary with species, and would alter the relative levels of different species attaching to solid surfaces (Chapter 3). This, in turn, would change the nature of the developing biofilm.

Such differences in bacterial attachment with growth and environmental conditions have been indicated by several workers (Ellwood et al. 1982; Wardell & Brown, 1980). Carbon-limited and carbon-sufficient conditions in continuous culture, not only affected the population constituents in the liquid phase, but also the nature of the developing biofilm on glass slides in enrichment cultures from the River Tay. A complex periphytic community with little associated polymer was produced by carbon-limited conditions, while carbon-sufficiency in the medium caused fewer bacterial numbers and species to attach to the surfaces though there were copious amounts of polymer present. The type of

limiting nutrient, e.g. glycerol or acetate, and other environmental parameters such as NaCl concentration not only affected the proportions of species in the liquid phase but totally changed the community make-up and numbers on the surface (Ellwood et al. 1982; Wardell & Brown, 1980). Corpe (1974a) found that glass slides submerged at various sites in the sea had different rates of bacterial attachment and also different growth rates on the surface. This was explained by relating the effect to nutrient levels in the water and other environmental parameters. In another environment, bacteria have been reported to vary their adsorption to soil seasonally (Novogradski, cited in Daniels, 1972). Thus there is evidence that the attachment of bacteria in natural environments is affected by nutrient and environmental conditions.

4.5 SUMMARY

1) Large changes in the surface characteristics of bacteria were produced by changing the culture conditions, e.g. carbon source or limiting nutrient and the growth rate of the bacteria as shown by Figure 4.2 to 4.7 and Table 4.7-4.10.

2) The extent and nature of the variability differed for each of the bacteria investigated, Pseudomonas fluorescens, Enterobacter cloacae, a Chromobacterium sp, and a Flexibacter sp.

3) The basis for charge and hydrophobic differences in the cell surface is proposed to be the result of changes in the quantities and/or arrangements of cell wall macromolecules, such as proteins, glycoproteins and lipids. These have been shown in the literature to vary with culture condition and dilution rate. The type and extent of polymer produced by bacteria is also proposed as an influence on cell surface characteristics, and was indicated as varying with culture conditions (Table 4.2 and the literature) and growth rate in the literature.

4) These differences in cell surface characteristics were reflected

in changed levels of bacterial attachment to solid surfaces. The levels of attachment to the two solid surfaces, i.e. the PD and TCD surfaces, were independently affected by alterations in bacterial surface characteristics. The attachment interaction was different for each bacterium investigated.

5) Attachment of Flexibacter sp, to solid surfaces may be an active process, since its attachment increased with time at all but the lowest dilution rate, and with growth rate. None of the other bacteria showed this consistent relationship. The Flexibacter sp, was also capable of a gliding motility across solid surfaces.

6) Changing nutrient and environmental conditions in aquatic environments will result in changes in the cell surface characteristics of the free-living bacteria. This in turn may alter the relative levels of different species attaching to solid surfaces and thence the nature of the developing biofilm.

CHAPTER FIVE

PHYSICO-CHEMICAL FACTORS AFFECTING BACTERIAL ATTACHMENT TO SURFACES

5.1 AIMS

To determine the physico-chemical factors affecting bacterial attachment to solid surfaces, with particular emphasis on diffuse electrical double layer effects, surface energy effects and the influence of hydrophobic interactions.

5.2 INTRODUCTION

Bacterial adsorption is largely governed by physico-chemical phenomena involving both long range and short range forces (Chapter 1). Given the complexities and variabilities of the interactions, an attempt has been made in this Chapter to investigate individual physico-chemical influences, and thence to build up a picture of the major interactions in bacterial attachment.

One of the prime components of the long range physico-chemical forces that affect bacterial adsorption to solid surfaces are electrostatic interactions. At normal physiological pH's bacteria bear a predominantly negative charge (Harden & Harris, 1953). If the bacteria are approaching a positively charged surface the net effect would be attractive. However, the majority of 'clean' surfaces are also negatively charged and the resultant interaction is repulsive. The extent of the repulsive effect will depend on the charge size of the surfaces and the distance between the two surfaces. Due to their net negative charges the surfaces will attract cations while tending to exclude anions. The cations form a diffuse layer near the surface, termed the diffuse electrical double-layer. The thickness ($1/K$) of this double-layer varies with the electrolyte concentration of the surrounding medium and the valency of the electrolyte. In general, with increasing electrolyte concentration and/or increasing electrolyte valency, the diffuse

electrical double-layer becomes more compact, i.e. the thickness decreases. The diffuse electrical double-layer thickness ($1/K$) can be calculated for symmetrical electrolytes in aqueous solution as

$$K = 0.327 \cdot 10^8 Zc^{1/2}$$

where Z is the valency and c the molar concentration of the electrolyte (Shaw, 1970).

Bacteria are colloidal sized particles and as such their interactions may be predicted by the DLVO theory (Shaw, 1970). In this theory the potential energy of interaction between two approaching particles is estimated not only by the electrical repulsion energies in their overlapping double-layers but also by the attraction energies of London-van der Waals forces. The balance between these two forces varies with interparticulate distance and the concentration and valency of electrolytes. The attraction energies, i.e. London-van der Waals forces, are unaltered by changes in electrolyte composition, while the repulsion energies vary with double-layer thickness. At low electrolyte concentration and larger $1/K$ there is an area of net attraction close to the surface, the primary minimum, but the repulsion energy at greater distances from the surface is very large and may be virtually insurmountable for a bacterium approaching the surface (Fig. 5.1). However, at small $1/K$, i.e. high electrolyte concentration and/or valency, another point of net attraction develops, the secondary minimum. This point of net attraction is at wider interparticulate distance than the primary minimum, and the two, i.e. the secondary and primary minima, are separated by an area of repulsive energy which is possibly too great for the bacterium to overcome (Fig. 5.2).

Marshall et al. (1971b) suggested that the reversible stage of bacterial attachment is caused by the weak adsorption of the bacteria at the secondary minimum. This has also been proposed for interactions between negatively charged tissue cells and their association with

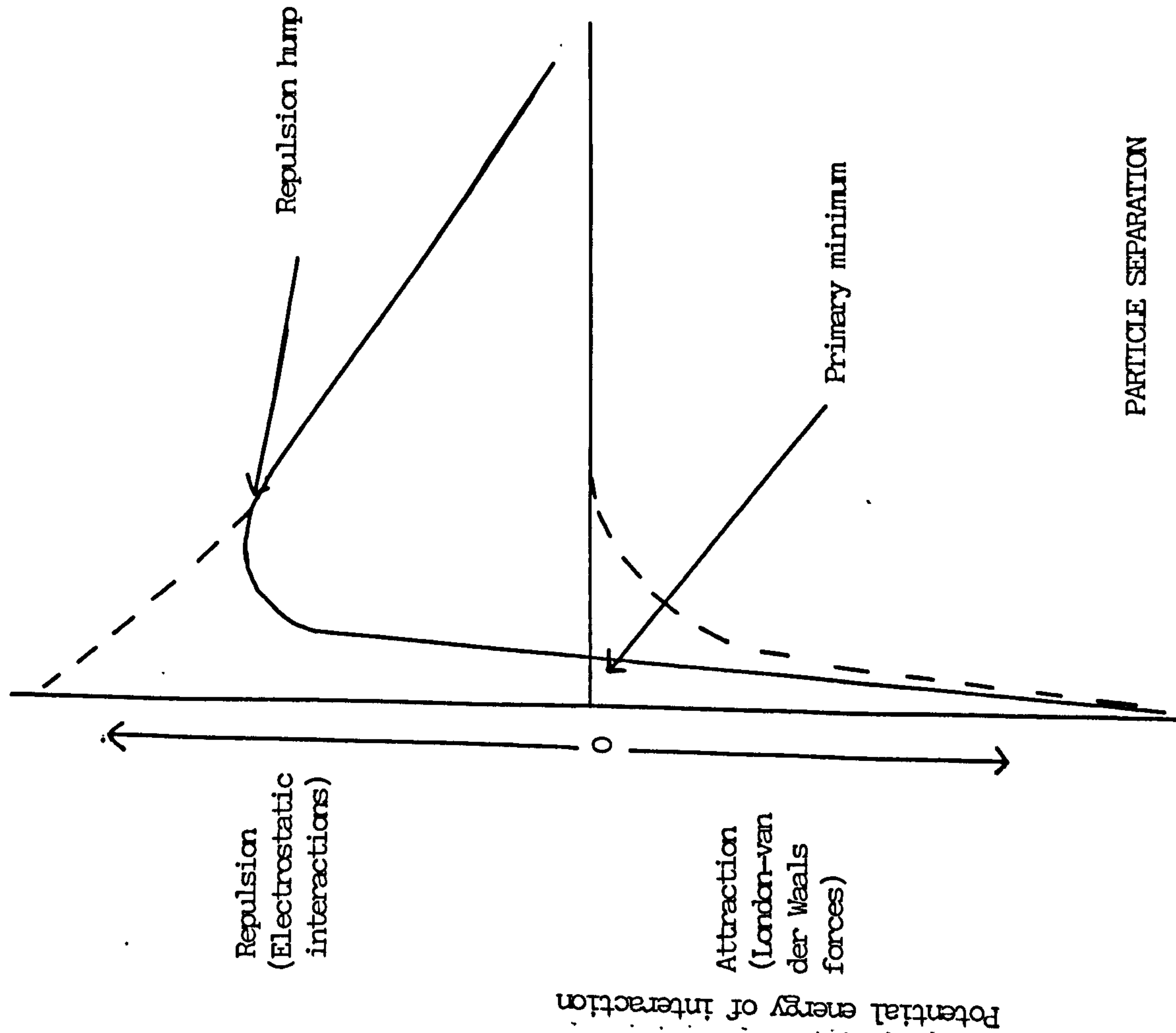


Figure 5.1 Potential energy of interaction between two particles at large $1/K$. The dotted lines represent the attractive and repulsive energies, the solid line represents the resultant energy of interaction

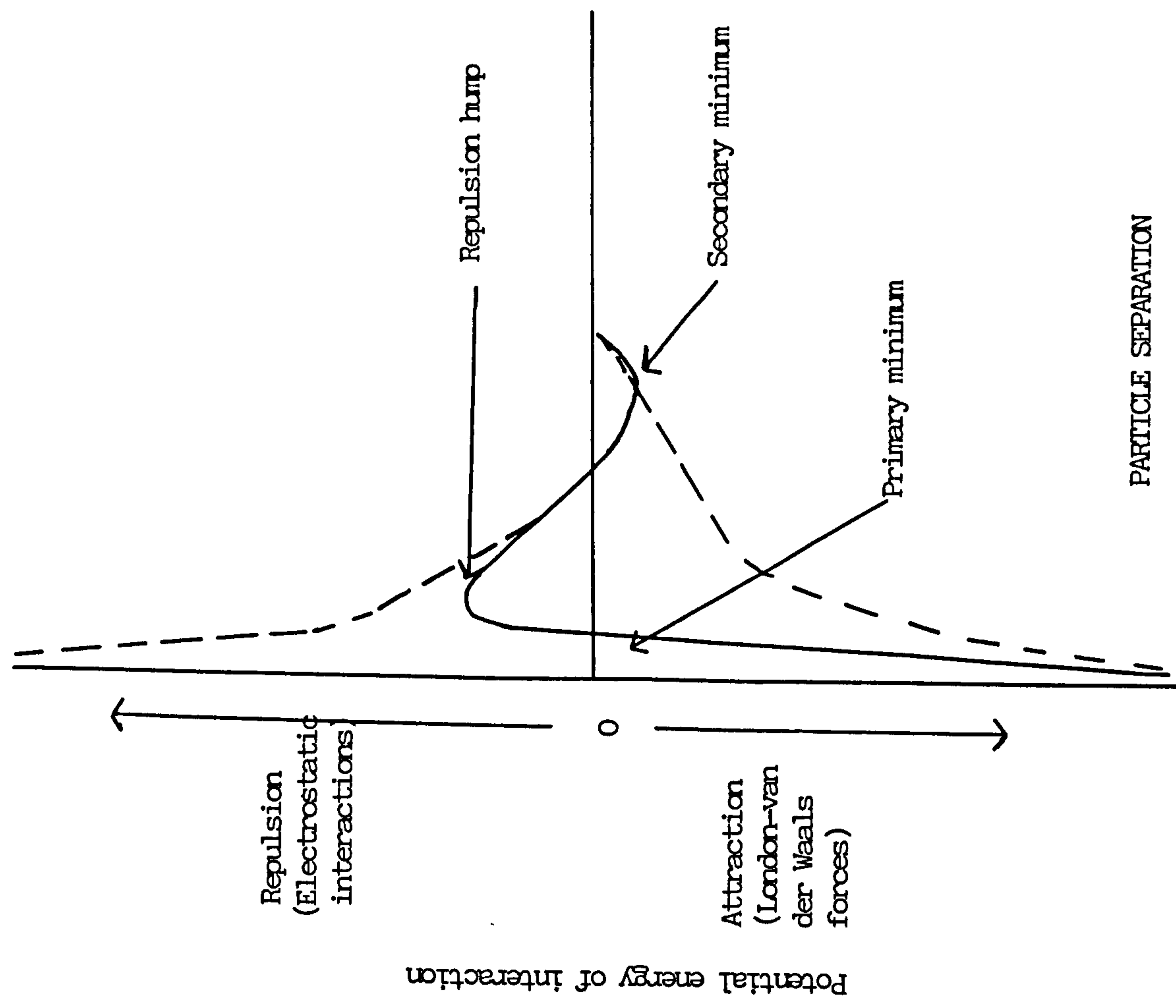


Figure 5.2 Potential energy of interaction between two particles at small $1/K$. The dotted lines represent the attractive and repulsive energies, the solid line represents the resultant energy of interaction

negatively charged surfaces (Curtis, 1967; Maroudas, 1975a).

Since the size of the diffuse electrical double-layer can be changed by altering electrolyte concentration and/or valency, it was possible to investigate the effect of $1/K$ on the permanent, irreversible attachment of the P. fluorescens (H_2), E. cloacae (H_{12}), the Chromobacterium sp, (H_{31}) and the Flexibacter sp (H_{38}).

The surface free energy of a substance, solid or liquid, is a measure of the total surface molecular groups free to interact with the surface groups of adjacent phases, i.e. surface free energy is a measure of the unsatisfied bonding capacity of a surface (Andrade, 1973). There are a number of possible short range interaction phenomena involving surface groups, molecules or atoms of adjacent phases,

- 1) van der Waals dispersion interactions which are weak interactions caused by fluctuations in the spatial concentration and distribution of electrons in molecules or atoms,
- 2) electrostatic interaction between charged groups,
- 3) dipole interactions, i.e. polar interactions between dipole-dipole, dipole-induced dipole and dipole-ion,
- 4) chemical bonding e.g. ionic, covalent, hydrogen bonding,
- 5) hydrophobic bonding.

Surface energies apply to interactions which occur when surfaces of adjacent phases are in molecular contact; they involve consideration of interactions at the primary minimum (Gerson & Zajic, 1979) and are probably the interactions of irreversible bacterial adsorption.

The free energy of a system tends towards a minimum; thus adsorption of substances onto a solid surface and the lowering of its potential bonding capacity, i.e. free energy, is energetically favoured. In an aqueous medium, the potential bonding capacity of the liquid and solid surface will, then, be partially satisfied since molecules from

the liquid phase will adsorb. The remaining residual bonding capacity is described by the free energy at the solid surface-liquid interface, i.e. the interfacial free energy. Generally, the larger the difference in surface energies between two phases, then the larger the interfacial energies.

Bacterial adsorption onto a solid surface in a liquid medium will only be favoured if there is a net reduction in the free energy of the system. The interactions in the system can be described by the equation:

$$\Delta G = \gamma_{SB} - \gamma_{SL} - \gamma_{BL} = \Delta F^{adh}$$

where ΔG is the free energy of the system and γ_{SB} , γ_{SL} and γ_{BL} are the interfacial energies of the surface-bacterium, surface-liquid and bacterium liquid respectively. ΔF^{adh} is the free energy of adhesion and when negative bacterial adhesion is favoured (Neumann et al. 1979).

ΔF^{adh} was calculated by Neumann et al. (1979) for cell adhesion to various substrata from a suspending medium, as a function of the surface tension (γ_{SV}) of the substratum. Three separate cases were described:

$$(1) \gamma_{LV} > \gamma_{BV}$$

where γ_{LV} is the surface tension of the suspending medium and γ_{BV} is the interfacial tension between the bacterium and vapour. In this case ΔF^{adh} was found to increase with increasing γ_{SV} . Since negative values of ΔF^{adh} favour bacterial adhesion, as γ_{SV} increases with a concomitant rise in ΔF^{adh} a decrease in bacterial adhesion is predicted.

$$(2) \gamma_{LV} < \gamma_{BV}$$

In this case ΔF^{adh} will decrease with increasing γ_{SV} , resulting in bacterial adhesion being favoured.

$$(3) \gamma_{LV} = \gamma_{BV}$$

ΔF^{adh} becomes zero and bacterial attachment becomes independent of γ_{SV} .

Thus a thermodynamic model predicts that attachment will increase with increasing surface tension of the solid substratum (γ_{SV}) if the

surface tension of the liquid (γ_{LV}) is lower than the surface tension of the cells (γ_{BV}). If the surface tension of the liquid (γ_{LV}) is larger than that of the bacteria (γ_{BV}) bacterial attachment will decrease.

An estimate of solid surface, free energies is commonly made by contact angle measurements. Thomas Young in 1805 resolved the equilibrium surface forces between a solid, liquid and vapour phase obtaining an equation:

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos\theta$$

where θ is the contact angle of a liquid on a solid, and γ_{SV} , γ_{SL} and γ_{LV} are the solid/vapour, solid/liquid and liquid/vapour interfacial tensions respectively.

Zisman (1964) developed a method for estimating solid surface energies by measuring the contact angles (θ) of a homologous series of liquids, with a range of surface tensions, on a solid surface. Plots of $\cos\theta$ against the liquid surface tension give a straight line relationship, the intercept with $\cos\theta = 1$ giving the critical surface tension (γ_c) of a solid surface. γ_c is indirectly related to surface free energy, and is defined as the maximum surface tension permitting spontaneous spreading of a liquid on a solid.

However, there are limitations to estimates of γ_c , since different liquids can give different straight line plots and hence different γ_c values, e.g. γ_c for a homologous series of non-polar liquids and for a homologous series of polar liquids will be different (Kitazakii and Hata, 1972). Thus the liquids must be carefully selected, if possible reflecting the same bonding potential as the substratum, e.g. polar, H-bonding. This obviously presents practical difficulties especially in relation to a bacterial surface; also in bacterial systems it is difficult to know how cell surfaces will be affected by a series of organic liquids in the estimate of γ_c .

Neumann et al (1974) developed a method by which the surface free energy of a solid can be determined from that of a single liquid of known surface tension on the solid. The evaluation of surface free energy is made from an empirically derived equation. Estimates of surface free energies of cells were made by Neumann et al (1979) using saline drops, the procedure followed in this investigation.

To investigate the applicability of the thermodynamic model for bacterial attachment (Neumann et al 1979) the liquid surface tension (γ_{LV}) of distilled water ($\gamma_{LV} = 7.28 \mu\text{J}/\text{cm}^2$) was lowered to selected values by adding DMSO (Table 5.8). The effect of the different liquid surface tensions on the attachment of the four freshwater bacteria, which themselves had different surface tensions (calculated from saline contact angle (θ_s) measurements by the Equation of State Approach, Neumann et al 1974) to PD and TCD surfaces was examined. PD has a lower surface tension ($2.84 \mu\text{J}/\text{cm}^2$) than TCD ($4.72 \mu\text{J}/\text{cm}^2$), so attachment to TCD should increase over PD when the liquid surface tension dropped below that of the cells.

A short range force which has recently been suggested as important in many different bacterial adsorption interactions, is hydrophobic bonding. This involves the interaction of non-polar groups on opposing surfaces. The hydrophobic interaction is thought to be attributable to the exclusion of non-polar groups from water, due to the strong attraction between molecules of water rather than to attraction forces between non-polar groups themselves. There are weak interactions between the non-polar, non-ionic groups, such as van der Waals forces, but the major driving force for hydrophobic bond formation is the involvement of water (Tanford, 1979; Kushner, 1978; Lewin, 1974).

The relationship between cell surface hydrophobicity and bacterial attachment was investigated in two ways, the first involved the use of HIC and the second the use of θ_s measurements of bacterial cells. The HIC

method supplies only a comparative estimate of cell surface hydrophobicity, i.e. the larger the proportion of cells retained in the gel, the more hydrophobic the bacterium. However, the estimate cannot be used to describe parameters such as the γ_c of the cells. Measurement of θ_s of cells, however, does give a quantitative estimate of the cell surface hydrophobicity, i.e. the larger θ_s the more hydrophobic the cell surface, and can be used to define other surface parameters such as the critical surface tension (γ_c) of the bacteria.

The HIC and θ_s measurements of the four bacteria shown in Chapter 4 were statistically analyzed in order to determine if there was a correlation between increasing cell surface hydrophobicity and increasing levels of attachment (60 mins) to PD and/or TCD.

The type of physico-chemical interactions between the solid surfaces and the bacteria were investigated further by determining the efficiency of a range of pH solutions, chemical agents, and detergents on detaching apparently permanently attached bacteria.

5.3 MATERIALS AND METHODS

5.3.1 Organisms

The four bacterial species described in Chapter 4, Pseudomonas fluorescens (H_2), Enterobacter cloacae (H_{12}), a Chromobacterium sp, (H_{31}) and a Flexibacter sp, (H_{38}) were used for investigation.

5.3.2 Culture Conditions and Inoculation Procedure

(a) Batch Culture

i) Minimal media: The culture conditions and minimal media described in Chapter 4, Section 4.2.2(a) were used for all four bacteria under investigation. The inoculation procedures were identical to those described in Chapter 4, as were the growth conditions, i.e. 15°C on a rotary incubator set at 150 rpm. The P. fluorescens, E. cloacae, and the Flexibacter sp. were grown in each condition for 24 hrs only, the

the Chromobacterium sp, for 24 and 48 hrs.

ii) PYE medium: The organisms were grown in pure culture in PYE medium (Chapter 2, Section 2.3.2) and stored at 4°C (Section 2.3.2). One ml aliquots of the stored stock cultures were inoculated into 150 ml of PYE medium, which was then incubated at 15°C on a rotary incubator at 150 rpm for 16 hrs.

b) Continuous Culture.

The culture conditions and inoculation procedure described in Chapter 4, Section 4.3.2b(i), was used to grow the bacteria at controlled dilution rates, i.e. growth rates.

(c) Growth in Electrolyte/PYE media.

To determine the effect of a selection of electrolyte concentrations on bacterial growth, an adaptation of the PYE broth (Section 2.3.2) medium was used. PYE medium broth (0.1% (w/v) peptone, 0.07% (w/v) yeast extract) was prepared with the addition of electrolytes to the required molarity, i.e. 0.M (control), 0.01 and 0.1M NaCl (Fisons Scientific Reagents, Loughborough); 0.01M and 0.1M MgCl₂ (May & Baker Ltd, London); and 0.1M AlCl₃ (Fisons Scientific Reagents, Loughborough); and 0.01M HEPES (Sigma Chemical Company, Poole) adjusted to pH 7.4 by 1M NaOH (BDH Chemicals Ltd, Poole). 3 ml quantities of the electrolyte media were independently placed in 5 ml screw cap bottles and autoclaved (15 min, 121°C).

Pure bacterial PYE stock cultures were then used to inoculate (100 µl inoculum) duplicate tubes of each electrolyte concentration medium. After growth at 15°C for 16 hrs, an estimate of the extent of growth was made by reading the absorbance of the cells and media at 540 nm (A_{540}) in a spectrophotometer (Unicam Instruments, Cambridge). An average reading of the duplicate values at each level of electrolyte concentration was calculated.

(d) Growth in Dimethyl Sulphoxide (DMSO)/PYE media.

To determine the effect of DMSO concentration on bacterial growth, PYE medium broth (0.1% (w/v) peptone, 0.07% (w/v) yeast extract) were prepared containing 0.01M HEPES buffer adjusted to pH 7.4 with 1M NaOH. This medium was sterilized by autoclaving (121°C, 15 min). To give a range of DMSO concentrations, the appropriate quantity of filter sterilized (0.45µ cellulose-acetate filter) DMSO solution (Fisons Scientific Reagents, Loughborough) was aseptically added to sterile 5 ml screw cap bottles followed by the addition of the PYE medium broth to a total volume of 3 mls. The final concentrations of DMSO were 0 (control), 3, 6, 9, 12, 15 and 18% (v/v).

The inoculation, growth and bacterial growth estimation procedure described in Section 5.3.2(c) were used for the DMSO media.

5.3.3 Attachment Assays

(a) Attachment Assays of Bacteria grown in Minimal Media and Continuous Culture.

The procedures described in Chapter 4, Section 4.3.3 were unaltered. Duplicate plates of PD and TCD were assayed at 5 and 60 mins for the chemostat experiments and 60 mins only for the minimal media experiments.

(b) Assays for the Effect of Electrolyte Concentration on Attachment.

Bacteria were grown in pure cultures in PYE medium (Section 5.3.2a(ii)) and were centrifuged at 10,960 av.g (Beckman model J-21B centrifuge) and washed once, to remove contaminating nutrients with 0.01M HEPES buffer, pH 7.4. The individual species were then resuspended to an optical density of 0.1 at 540 nm in a colorimeter (Corning Colorimeter 252) in a series of electrolyte solutions in 0.01M HEPES buffer (pH 7.4). The electrolyte concentrations were 0M (control), 0.01M and 0.1M NaCl, 0.01M and 0.1M MgCl₂ and 0.1M AlCl₃.

5 ml aliquots of the cell suspensions were then placed in PD and TCD petri dishes in duplicate. After 60 mins incubation at 15°C the

attached cells were stained and the levels of attachment were estimated in the usual manner (Section 2.3.4).

(c) Assays for the Effect of Surface Tension on Attachment.

The procedure described in Section 5.3.3(b) for the growth and sampling of bacteria was used. The cells were, however, resuspended to an optical density of 0.1 at 540 nm (Corning Colorimeter 252) in 0.01M HEPES buffer solutions (pH 7.4) containing DMSO. The DMSO concentrations were 0 (control), 3, 6, 9, 12, 15 and 18% (v/v).

5 ml aliquots of these solutions were transferred to duplicate PD and TCD petri dishes and incubated for 60 mins at 15°C. The remaining assay procedure was described above (Section 2.3.4).

5.3.4 Detachment Assays

The procedure for culture sampling by centrifugation and washing with 0.01M HEPES (pH 7.4) solution described for the attachment assays was repeated, as was the assay procedure for one hour attachment (Section 5.3.3(b)).

One set of duplicate plates for each bacterial species was then washed and stained (Section 2.3.4), after 60 mins attachment at 15°C. The remaining sets of duplicate plates were then washed three times with 0.01M HEPES solution (pH 7.4), to remove those bacteria not firmly attached to the test substrata. During the washing and treatment procedures the attached bacterial cells were always maintained under a covering of 0.01M HEPES (pH 7.4). At no time were the surfaces allowed to dry. The washed duplicate plates were then exposed to 5 ml aliquots of one of the following solutions;

- 1) 0.01 M HEPES buffer, pH 7
- 2) 0.01M HEPES buffer, pH 5
- 3) 0.01M HEPES buffer, pH 9
- 4) 0.1M NaCl in 0.01M HEPES buffer (pH 7)

5) 0.1M MgCl_2 in 0.01M HEPES buffer (pH 7)

6) 0.1% (v/v) sodium lauryl sulphate (SLS) (BDH Chemicals Ltd, Poole) in 0.01M HEPES buffer (pH 7).

7) 0.1% (v/v) RBS (Chemical Concentrates (RBS) Ltd, London) in 0.01M HEPES buffer (pH 7)

8) 0.1% (v/v) Tween 80 (Sigma Chemical Company, Poole) in 0.01M HEPES buffer (pH 7).

The attachment surfaces were then incubated for a further 60 mins at 15°C in the presence of the treatment solutions. At the end of this period the PD and TCD surfaces were washed, stained and levels of attachment were estimated (Section 2.3.4).

5.3.5 Contact Angle Measurements on Lawns of Cells

The method for the saline contact angle (θ_s) of lawns of cells has been described (Chapter 4, Section 4.3.4). Neumann et al's (1974) empirically derived equation for estimating critical surface tension (γ_c) and surface free energies of a solid from the contact angle of a liquid on the surface was used.

5.3.6 Contact Angle Measurement of PD and TCD Surfaces

The contact angle ($\theta_{\text{H}_2\text{O}}$) of a 10 μl drop of sterile distilled water (autoclaved 15 min, 121°C) was measured on clean PD and TCD surfaces using a vernier microscope with a goniometer eye piece. At least ten measurements were taken from each of two surfaces of PD and TCD. The contact angle measurements varied only slightly ($\pm 0.5^\circ$) and an average value of all the measurements was taken to represent the $\theta_{\text{H}_2\text{O}}$ of the solid surfaces. Using an empirically derived equation (Neumann et al. 1974) the critical surface tension (γ_c) and surface free energies of the solid surfaces were estimated.

5.3.7 Liquid Surface Tension Measurement (γ_{LV})

The liquid surface tension measurements of distilled water, 3, 6, 9, 12, 15 and 18% (v/v) DMSO solutions were measured by using a platinum loop and torsion balance (White Electrical Instruments Co, Ltd, England). To avoid contamination, the loop and glass sample dish were washed in concentrated nitric acid (Fisons Scientific Reagents, Loughborough) followed by several washes in distilled water between measuring γ_{LV} of the test solutions. The measurements on the DMSO solutions were performed in cool conditions ($\approx 10^{\circ}\text{C}$) to avoid any influence on γ_{LV} measurement of DMSO evaporation.

5.3.8 Hydrophobic Interaction Chromatography (HIC) of Bacterial Cells

The procedure for the interaction chromatography of cells has been described in Chapter 4, Section 4.3.5. Hydrophobic Interaction Chromatography (HIC) was used to determine if high cell surfaced hydrophobicity enhanced bacterial attachment to solid surfaces.

5.4 RESULTS

5.4.1 The Effect of Diffuse Electrical Double-Layer Thickness ($1/K$) (Electrolyte concentration) on Bacterial Attachment to PD and TCD Surfaces

There was no correlation between diffuse double-layer thickness and attachment for any of the species investigated (Tables 5.1 to 5.4). This was confirmed by correlation co-efficients calculated for the interaction between $1/K$ and bacterial attachment to both surfaces (Table 5.5). The only significant correlation was found for P. fluorescens attaching to the TCD surface, which showed a slight positive correlation i.e. attachment increased with increasing double-layer thickness. This was the opposite of the predicted interaction since theoretically the correlation should be negative, i.e. attachment should increase with decreasing $1/K$. (Section 5.2).

TABLE 5.1 The Effect of Electrical Double-Layer Thickness ($1/K$) on the Attachment of Pseudomonas fluorescens to PD and TCD Surfaces

Valency	Electrolyte (M) Concentration	Calculated $1/K$ (nM)	$A_{590}(\times 10^{-3})$ of attached bacteria	
			PD	TCD
0	0	Approaching Infinity	21 (± 0.8) ^a	42 (± 6)
UNIVALENT	10^{-2}	3.1	35 (± 3)	47 (± 6)
DIVALENT	10^{-2}	1.5	25 (± 4)	43 (± 9)
UNIVALENT	10^{-1}	1.0	40 (± 11)	41 (± 2)
DIVALENT	10^{-1}	0.5	29 (± 5)	38 (± 2)
TRIVALENT	10^{-1}	0.3	53 (± 4)	28 (± 2)

a

Parentetical values represent 95% confidence limits of the mean
(n = 8)

TABLE 5.2 The Effect of Electrical Double-Layer Thickness (1/K) on the Attachment of Enterobacter cloacae to PD and TCD Surfaces

Valency	Electrolyte (M) Concentration	Calculated 1/K (nM)	A ₅₉₀ (x10 ⁻³) of attached bacteria	
			PD	TCD
0	0	Approaching Infinity	47 (± 3) ^a	56 (± 4)
UNIVALENT	10 ⁻²	3.1	49 (± 2)	50 (± 5)
DIVALENT	10 ⁻²	1.5	32 (± 3)	34 (± 4)
UNIVALENT	10 ⁻¹	1.0	43 (± 3)	45 (± 4)
DIVALENT	10 ⁻¹	0.5	33 (± 3)	52 (± 3)
TRIVALENT	10 ⁻¹	0.3	34 (± 0.8)	36 (± 4)

^a Parenthetical values represent 95% confidence limits of the mean (n=8)

TABLE 5.3 The Effect of Electrical Double-Layer Thickness ($1/K$) on the Attachment of the Chromobacterium sp. to PD and TCD Surfaces

Valency	Electrolyte (M) Concentration	Calculated $1/K$ (nM)	$A_{590}(\times 10^{-3})$ of attached bacteria	
			PD	TCD
0	0	Approaching Infinity	27 (\pm 5)	27 (\pm 4)
UNIVALENT	10^{-2}	3.1	22 (\pm 2)	27 (\pm 2)
DIVALENT	10^{-2}	1.5	18 (\pm 3)	20 (\pm 3)
UNIVALENT	10^{-1}	1.0	14 (\pm 6)	17 (\pm 0.8)
DIVALENT	10^{-1}	0.5	11 (\pm 3)	22 (\pm 3)
TRIVALENT	10^{-1}	0.3	68 (\pm 7)	57 (\pm 4)

^a Parenthetical values represent 95% confidence limits of the mean (n= 8)

TABLE 5.4 The Effect of Electrical Double-Layer Thickness ($1/K$) on the Attachment of the Flexibacter sp. to PD and TCD Surfaces

Valency	Electrolyte (M) Concentration	Calculated $1/K(\text{nM})$	$A_{590}^{\text{PD}} (\times 10^{-3})$ of attached bacteria	TCD
0	0	Approaching Infinity	164 (± 5)	52 (± 7)
UNIVALENT	10^{-2}	3.1	105 (± 7)	50 (± 10)
DIVALENT	10^{-2}	1.5	33 (± 3)	22 (± 3)
UNIVALENT	10^{-1}	1.0	23 (± 0.8)	18 (± 2)
DIVALENT	10^{-1}	0.5	33 (± 4)	37 (± 8)
TRIVALENT	10^{-1}	0.3	97 (± 7)	73 (± 7)

^a Parenthetical values represent 95% confidence limits of the mean ($n = 8$)

TABLE 5.5 The Correlation Co-efficients (r) for the Relationship
between Electrical Double-Layer Thickness (1/K) and Bacterial
Attachment to the PD and TCD Surfaces

Bacterial Species	PD	TCD
<i>Pseudomonas fluorescens</i>	- 0.339	0.825
<i>Enterobacter cloacae</i>	0.752	0.277
<i>Chromobacterium</i> sp	- 0.334	- 0.326
<i>Flexibacter</i> sp	0.403	0.096

Values of r greater than 0.811 differ significantly from 0 at 5% probability level

r was calculated for 1/K values between 3.1 to 0.3 nM,

The salt solutions were shown to affect the growth levels of the four bacterial species (Table 5.6). However, there was no apparent relationship between growth levels and the bacterial attachment in the salt solutions.

5.4.2 The Effect of Liquid Surface Tensions on Bacterial Attachment to PD and TCD Surfaces

The DMSO solutions gave a range of γ_{LV} from 6.5 to 7.2 $\mu\text{J}/\text{cm}^2$ (Table 5.7) while the γ_c of the bacterial cells varied from 6.83 to 7.15 $\mu\text{J}/\text{cm}^2$ (Table 5.8). The only bacterium which underwent considerable changes in attachment with liquid surface tension (γ_{LV}) was Enterobacter cloacae (Fig. 5.3). Attachment to both PD and TCD decreased with decreasing γ_{LV} , the drop occurring at γ_{LV} values below the critical surface tension (γ_c) of the cells. The attachment interaction with the two surfaces also changed. The attachment to TCD did not fall as fast as that to PD with lowering γ_{LV} , and as a consequence, although attachment to both surfaces was similar at the higher values of γ_{LV} , at lower γ_{LV} the attachment to TCD was greater.

The attachment of Pseudomonas fluorescens, and the Chromobacterium sp (Figs. 5.4 and 5.5) decreased slightly with decreasing liquid surface tension (γ_{LV}) of the suspending solution. There was no major change in attachment to either solid surface when the liquid surface tension dropped below γ_c of the cells. The relative proportions of cells attaching to each surface remained largely unaltered over the whole γ_{LV} range, e.g. P. fluorescens attachment to TCD was considerably higher than that to PD at most γ_{LV} (Fig. 5.4). The attachment of the Flexibacter sp (Fig. 5.6) changed little with γ_{LV} although attachment to the PD surface rose in a medium with γ_{LV} of 6.5 $\mu\text{J}/\text{cm}^2$. However, this result was not repeated (Appendix Table 15). Again there was little difference in attachment levels between γ_{LV} greater than cell surface tension and γ_{LV} less than cell γ_c . At all γ_{LV} values, numbers of cells attaching to

TABLE 5.6 The Effect of Electrolyte Concentration (related to 1/K)
on the Growth of Four Bacterial Species

Electrolyte (M) Concen- tration	Calculated 1/K (nM)	P. fluorescens	A540 of Growth E. cloacae	Chromobacterium sp	Flexibacter sp
Control 0	-	0.4	0.4	0.6	0.097
Na ⁺ 10 ⁻²	3.1	0.55	0.303	0.079	0.175
Mg ²⁺ 10 ⁻²	1.5	0.63	0.378	0.317	0.296
Na ⁺ 10 ⁻¹	1.0	0.637	0.228	0.11	0.204
Mg ²⁺ 10 ⁻¹	0.5	0.431	0.402	0.193	0.099
Al ³⁺ 10 ⁻¹	0.3	0.117	0.126	0.145	0.099

TABLE 5.7 Measured Liquid Surface Tension of Dimethyl Sulfoxide (DMSO) Solutions

% DMSO (v/v) H ₂ O	0	3	6	9	12	15	18
Liquid Surface Tension ($\mu\text{J}/\text{cm}^2$)	7.2	7.1	6.9	6.8	6.7	6.6	6.5

TABLE 5.8 Solid Surface Tension of Bacterial Cells Estimated from Water Contact Angles on Lawns of Cells by the Equation of State Approach^a

Bacterial Species	Water Contact Angle Cells ($^{\circ}$)	Critical Surface Tension ($\mu\text{J}/\text{cm}^2$)
Pseudomonas fluorescens	12	7.05
Enterobacter cloacae	11	7.07
Chromobacterium sp	7.5	7.15
Flexibacter sp	19	6.83

^aNeumann et al. (1974)

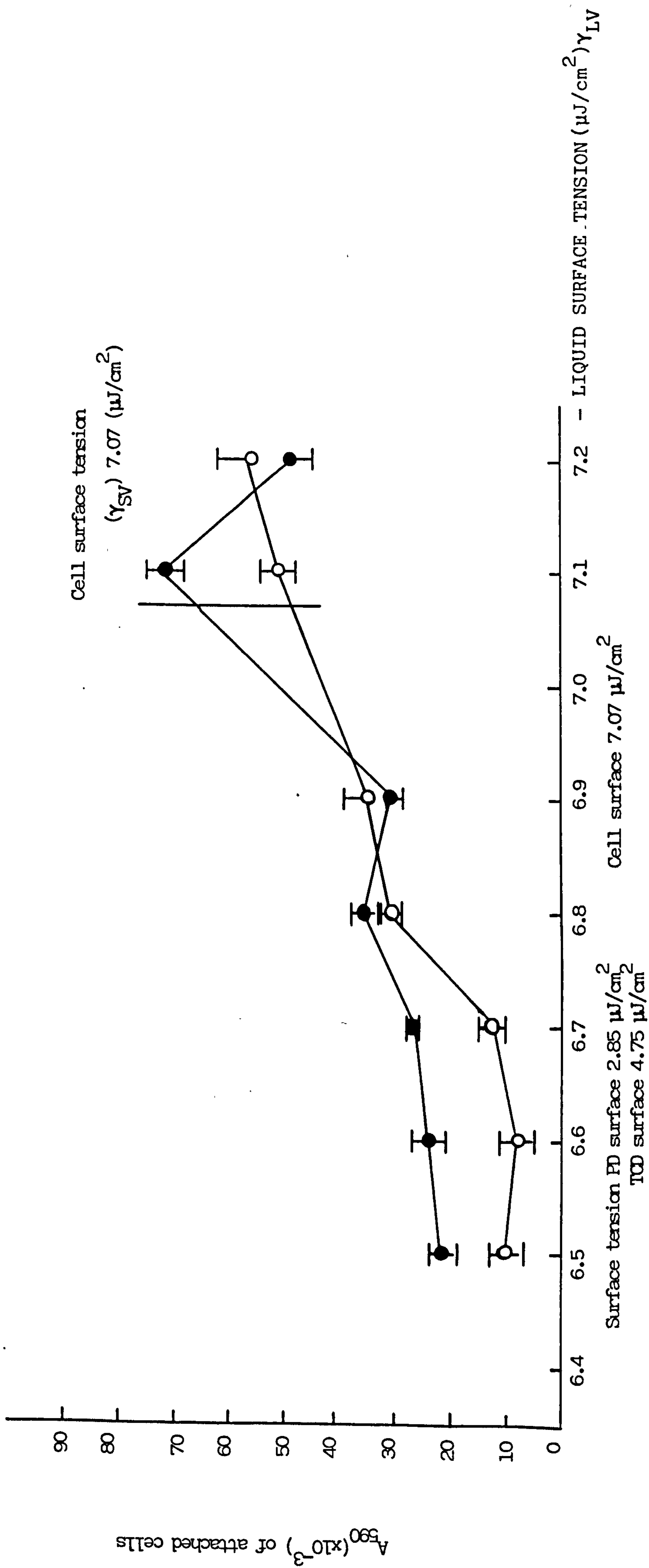


Figure 5.3 The effect of liquid surface tension (γ_{LV}) on the attachment of Enterobacter cloacae to PD and TCD surfaces

(O), cells attached to PD surface; (●), cells attached to TCD surface; the line across the graph represents the bacterium's surface tension
The bars represent the 95% confidence limits of the mean (n = 8)

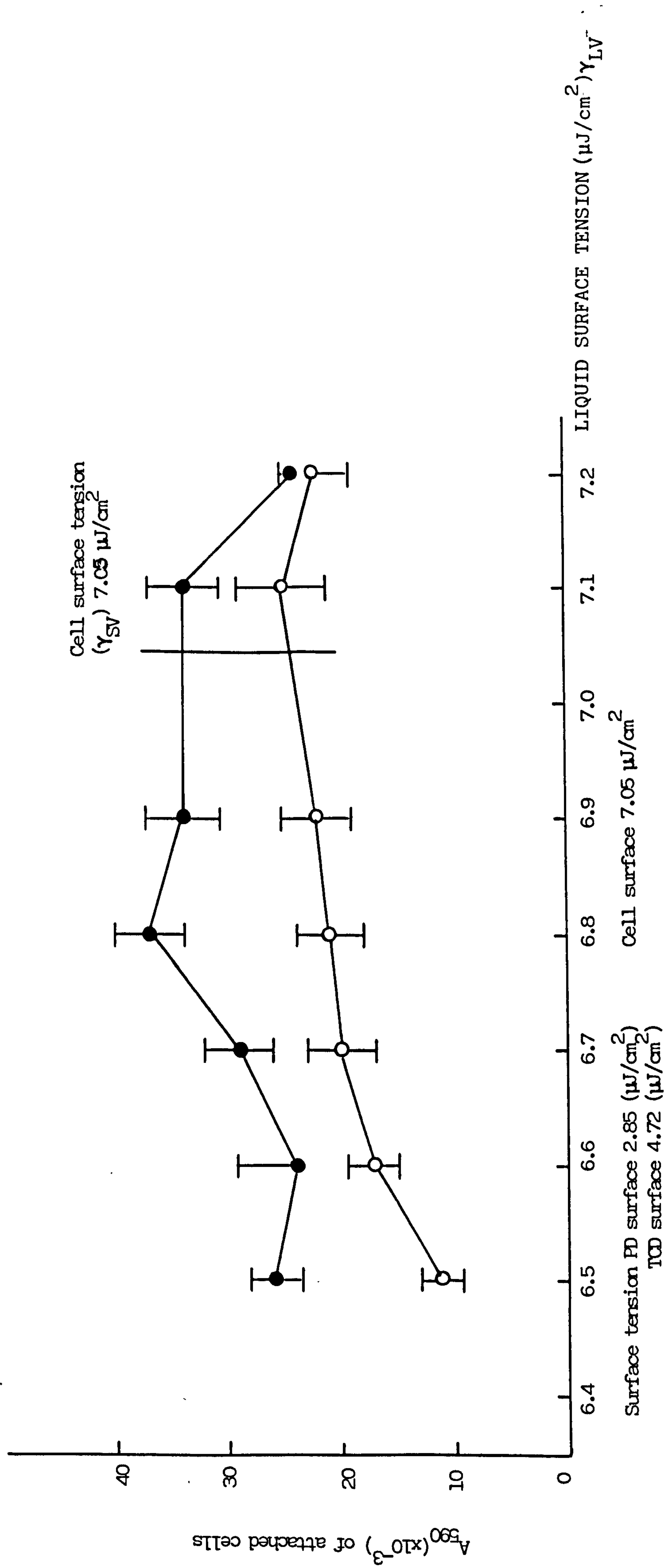


Figure 5.4 The effect of liquid surface tension (γ_{LV}) on the attachment of Pseudomonas fluorescens to PD and TCD surfaces.

(O), cells attached to PD surface; (●), cells attached to TCD surface; The bars represent the 95% confidence limits of the mean (n = 8); The line across the graph represents the bacterium's surface tension

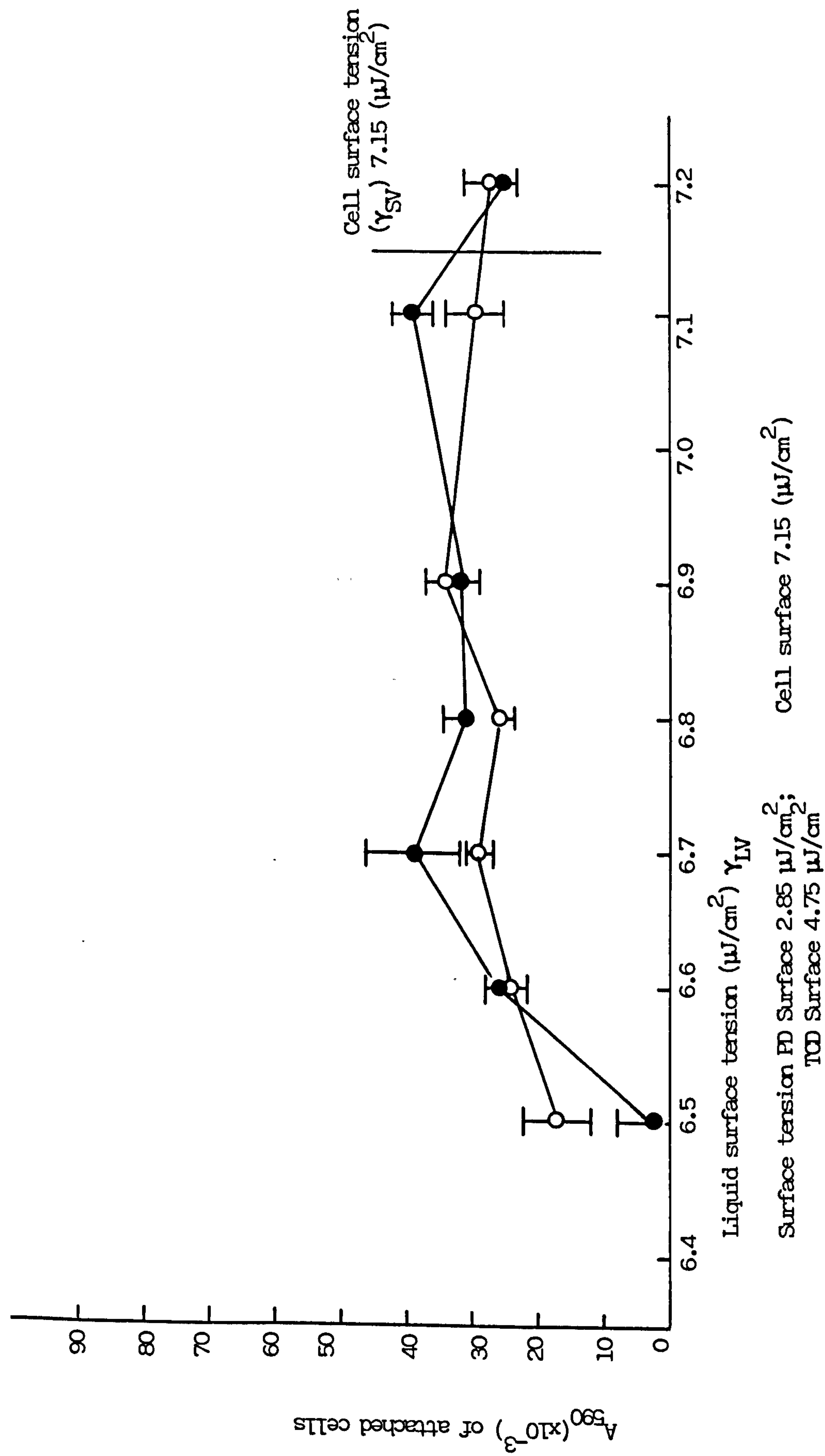


Figure 5.5 The effect of liquid surface tension (γ_{LV}) on the attachment of the Chromobacterium sp to PD and TCD surfaces. (O), cells attached to PD surface; (●), cells attached to TCD surface; the line across the graph represents the bacterium's surface tension

The bars represent the 95% confidence limits of the mean (n = 8)

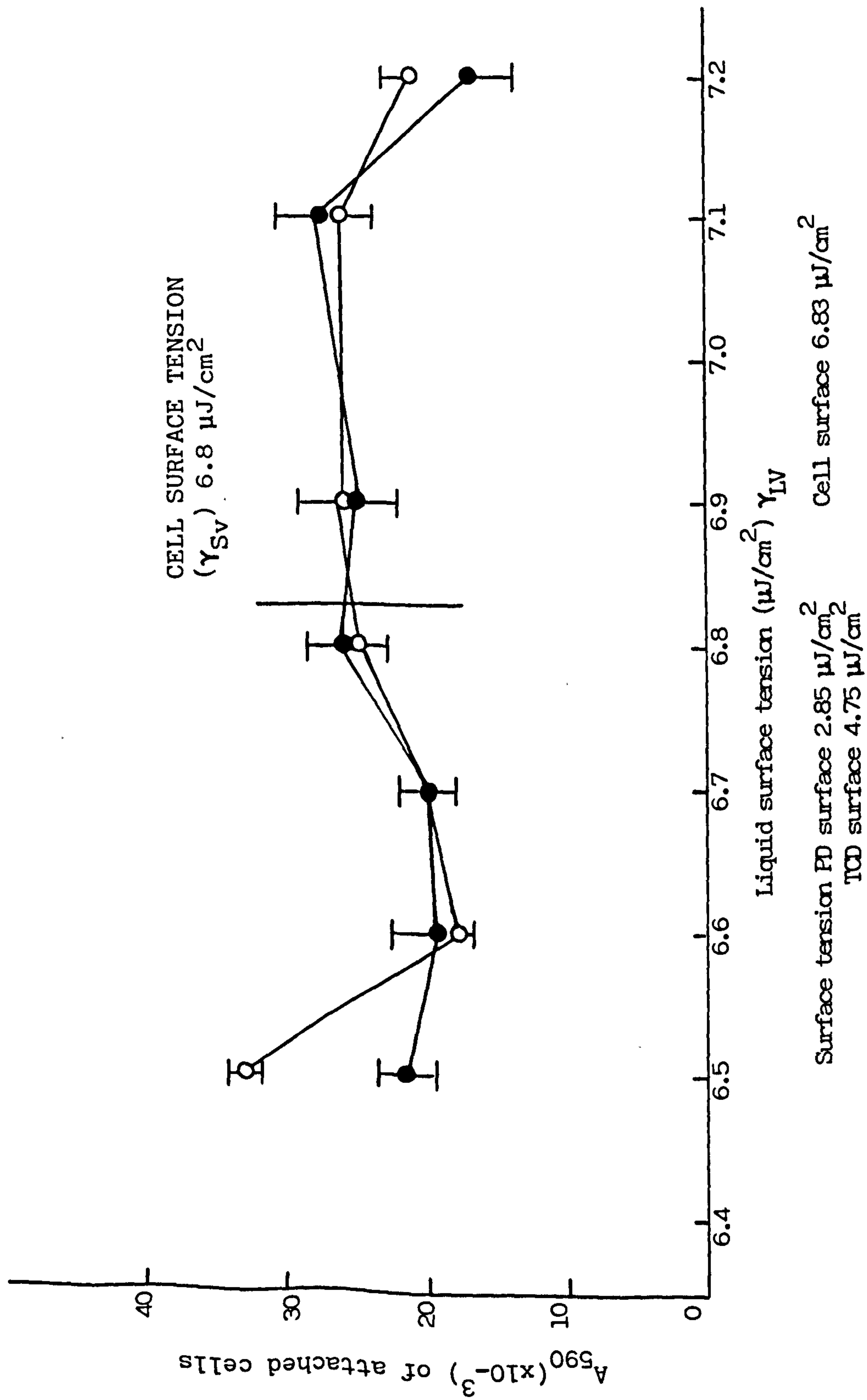


Figure 5.6 The effect of liquid surface tension (γ_{LV}) on the attachment of the Flexibacter sp to PD and TCD surfaces (O), cells attached to PD surface; (●), cells attached to TCD surface; the line across the graph represents the bacterium's surface tension

The bars represent the 95% confidence limits of the mean (n = 8)

each surface was similar.

DMSO had a large effect on bacterial growth which was inhibited with increasing concentrations of DMSO for all four bacteria (Fig. 5.7).

5.4.3 The Detachment of Attached Bacteria by Chemical and Environmental Treatments

The results of the detachment experiments indicate that none of the four freshwater bacteria investigated detaches to any great extent after 60 mins in buffered 0.01M HEPES solution at pH 5, 7 and 9 (Table 5.9). Nor do treatments of 0.1M NaCl and 0.1M MgCl₂ increase the likelihood of the bacteria detaching from the PD or the TCD surfaces (Table 5.10-5.13).

However, treatments with all three detergents affected the rates of bacterial detachment from the surfaces. The effects varied with the bacterial species investigated and with the surface to which the bacteria were attached. P. fluorescens detached markedly in all three detergents, though to a lesser extent in RBS, an anionic detergent. In all cases the effect of detachment was greatest to the PD surface (Table 5.10). The anionic detergent RBS had no effect on the detachment of E. cloacae (Table 5.11) from either surface. However, the neutral detergent Tween-80 increased bacterial detachment from both PD and TCD though to a greater extent from the PD surface. The effect of SLS, an anionic detergent caused the largest levels of detachment from PD and also smaller levels of detachment from the TCD surface. The Chromobacterium sp and the Flexibacter sp reacted identically to SLS and RBS. Levels of attachment to PD decreased for both species after treatment with these detergents. The attachment to the TCD surface was unaffected. The Chromobacterium sp showed similar detachment interactions with Tween-80, however, the Flexibacter sp showed no detachment from either surface in the presence of this detergent (Tables 5.12-5.13).

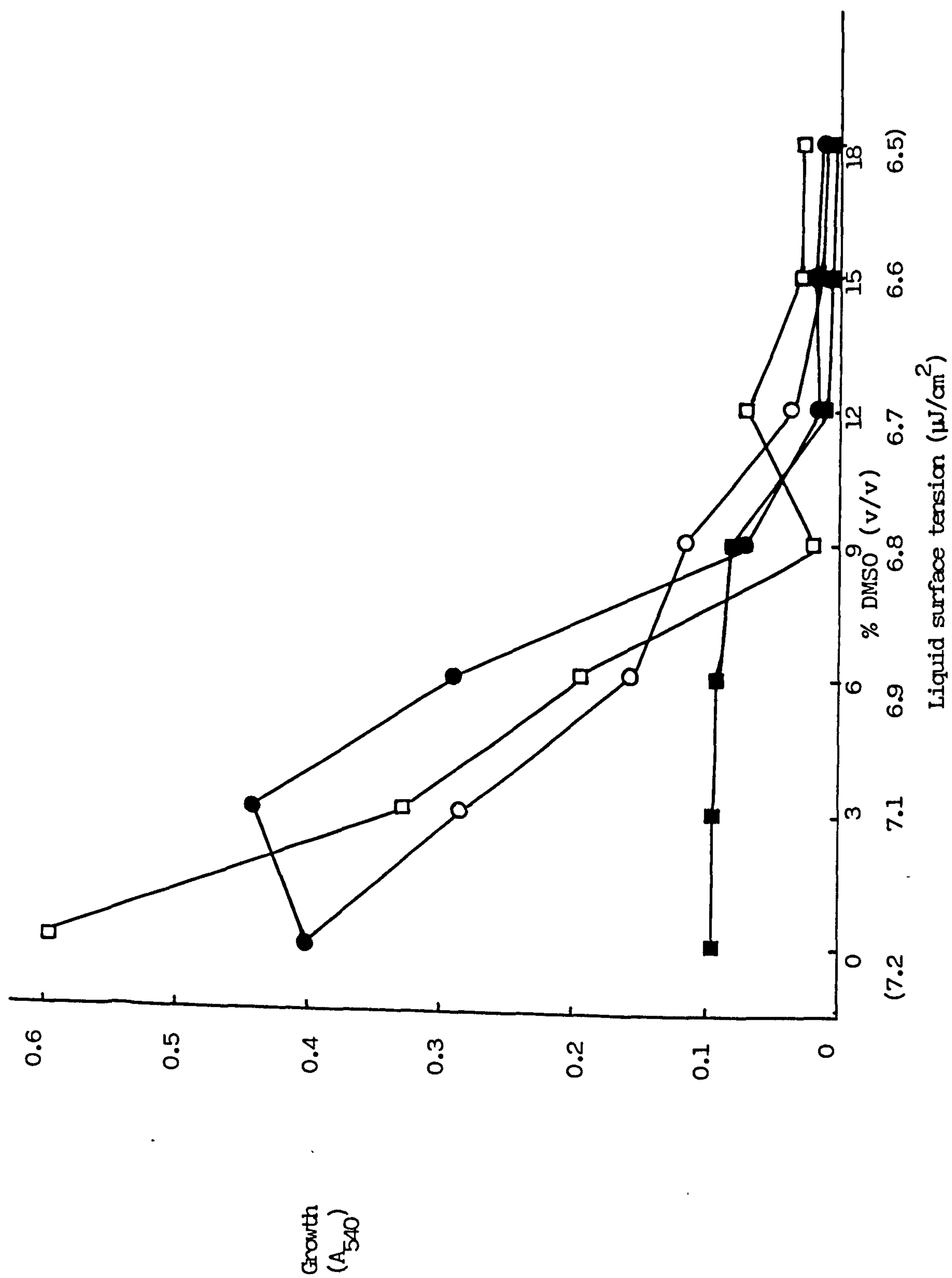


Figure 5.7 The effect of DMSO concentration (% v/v) (related to liquid surface tension) on the growth of four bacterial species. (●), *Pseudomonas fluorescens*; (○), *Enterobacter cloacae*; (□), *Chromobacterium* sp.; (■), *Flexibacter* sp.

TABLE 5.9 The Detachment of Four Bacterial Species after Different pH Treatments

pH	Pseudomonas fluorescens		Enterobacter cloacae ($\times 10^{-3}$)		Chromobacterium sp. attached cells		Flexibacter sp.	
	PD	TCD A_{590}	PD	TCD	PD	TCD	PD	TCD
Control (no detachment)	44(± 7) ^a	52(± 3)	27(± 2)	39(± 3)	35(± 4)	18(± 2)	14(± 3)	16(± 0.8)
5	48(± 6)	52 (± 4)	27(± 2)	39(± 5)	38(± 2)	16(± 3)	13(± 2)	16(± 0.8)
7	44(± 8)	50 (± 4)	34(± 3)	31(± 3)	45(± 8)	17(± 2)	10(± 0.8)	14(± 3)
9	58(± 8)	48 (± 7)	29(± 5)	41(± 4)	30(± 8)	16(± 0.8)	9(± 0.8)	14(± 8)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 5.10 The Detachment of P. fluorescens by Chemical Treatments

TREATMENT	$A_{590}(\times 10^{-3})$ attached cells	
	PD	TCD
Control (no detachment)	44 (± 7) ^a	52 (± 3)
1 hr detachment only	44 (± 8)	50 (± 4)
0.1M NaCl	39 (± 3)	47 (± 4)
0.1M MgCl ₂	39 (± 5)	51 (± 6)
0.1% (v/v) SLS	5 (± 0.8)	16 (± 3)
0.1% (v/v) RBS	15 (± 2)	42 (± 2)
0.1% (v/v) Tween-80	1 (± 0.8)	26 (± 2)

^a Parenthetical values represent 95% confidence limits of the mean

(n = 8)

TABLE 5.11 The Detachment of E. cloacae by Chemical Treatments

TREATMENT	$A_{590}(\times 10^{-3})$ attached cells	
	PD	TCD
Control (no detachment)	27 (\pm 2) ^a	39 (\pm 3)
1 hr detachment only	34 (\pm 3)	31 (\pm 3)
0.1M NaCl	34 (\pm 2)	37 (\pm 3)
0.1M MgCl ₂	33 (\pm 3)	41 (\pm 4)
0.1% (v/v) SLS	4 (\pm 2)	26 (\pm 3)
0.1% (v/v) RBS	27 (\pm 10)	34 (\pm 3)
0.1% (v/v) Tween-80	17 (\pm 4)	27 (\pm 6)

^aParenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 5.12 The Detachment of the Chromobacterium sp. by Chemical Treatments

TREATMENT	$A_{590}(\times 10^{-3})$ attached cells	
	PD	TCD
Control (no detachment)	35 (\pm 4) ^a	18 (\pm 2)
1 hr detachment only	45 (\pm 8)	17 (\pm 2)
0.1M NaCl	37 (\pm 4)	20 (\pm 3)
0.1M MgCl ₂	44 (\pm 3)	23 (\pm 0.8)
0.1% (v/v) SLS	8 (\pm 8)	22 (\pm 2)
0.1% (v/v) RBS	9 (\pm 2)	19 (\pm 0.8)
0.1% (v/v) Tween-80	8 (\pm 4)	17 (\pm 3)

^aParenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 5.13 The Detachment of the Flexibacter sp. by Chemical Treatments

TREATMENT	$A_{590}(\times 10^{-3})$ attached cells	
	PD	TCD
Control (no detach- ment)	14 (\pm 3) ^a	16 (\pm 0.8)
1 hr detachment only	10 (\pm 0.8)	14 (\pm 3)
0.1M NaCl	12 (\pm 3)	14 (\pm 0.8)
0.1M MgCl ₂	14 (\pm 0.8)	19 (\pm 2)
0.1% (v/v) SLS	5 (\pm 2)	17 (\pm 3)
0.1% (v/v) RBS	2 (\pm 0.8)	15 (\pm 2)
0.1% (v/v) Tween -80	12 (\pm 2)	18 (\pm 2)

^a Parenthetical values represent 95% confidence limits of the mean
(n = 8)

(Results of repeat experiments for the effect of double-layer thickness, liquid surface tension on bacterial attachment, and the detachment assays are described in Appendix Tables 14 to 16).

5.5 RESULTS OF STATISTICAL COMPARISONS

5.5.1 The Effect of Bacterial Cell Surface Hydrophobicity on Bacterial Attachment to PD and TCD Surfaces

The results presented in Chapter 4 (Table 4.7-4.10) showed no apparent relationship between bacterial hydrophobicity and levels of bacterial adsorption to either the hydrophobic (PD) surface or the relatively hydrophilic (TCD) surface (Table 5.14 for a physico-chemical description of the solid surfaces). This observation was supported by calculating the correlation co-efficients between cell surface hydrophobicity, measured by HIC, and attachment to PD and TCD surfaces for individual species (Table 5.15). The calculated correlation co-efficients show no significant positive or negative relationship between cell hydrophobicity and bacterial attachment.

The calculation of similar correlation co-efficients for the relationship between cell surface hydrophobicity measured by θ_s and bacterial attachment presents a slightly different picture (Table 5.16). These results indicated that for two of the four bacterial species, the Chromobacterium sp, and the Flexibacter sp, the hydrophobicity of the cell as measured by θ_s , had a significant positive relationship with bacterial attachment to PD and TCD surfaces, i.e. increased hydrophobicity is related to increased attachment. However, the relationship for these two species is not entirely consistent. The Chromobacterium sp's attachment to PD after 48 hrs growth in the minimal media, is the only experimental condition in which there was a significant correlation with hydrophobicity (Table 5.16) and the Flexibacter sp, showed no significant correlation between cell hydrophobicity and attachment to PD in one of the duplicate

TABLE 5.14 Water Contact Angle Measurements and Calculated Estimates of Parameters (Using Equation of State Approach)^a of PD and TCD Surfaces

	PD	TCD
Water Contact Angle (θ) ^o	90	59
Solid Surface Tension ($\mu\text{J}/\text{cm}^2$)	2.84	4.72
Solid-Liquid Interfacial Tension	28.4	10.2
Cosine of θ	0	0.52
Cosine $\theta \times$ solid surface tension	0	3.71
Good's Interaction Parameter	0.795	0.935
^b Surface Charge (Ncm^{-1})	5.6×10^{-4}	3.3×10^{-4}

^aNeumann et al. (1974)

^bFigures from Maroudas (1973)

TABLE 5.15 The Correlation Co-efficients (r) for the Relationship between Bacterial Cell Surface Hydrophobicity Measured by HIC and 60 min Attachment to PD and TCD Surfaces (Calculated from results presented in Tables 4.7 - 4.10)

Bacterial Species	PD	TCD
<i>Pseudomonas fluorescens</i>	0.637	0.521
<i>Enterobacter cloacae</i>	-0.043	0.129
<i>Chromobacterium</i> sp	0.530	0.828
<i>Flexibacter</i> sp	-0.881	-0.811

Values of r greater than 0.950 differ significantly from 0 at 5% probability level.

TABLE 5.16 The Correlation Co-efficient (r) for the Relationship
between Bacterial Cell Surface Hydrophobicity Measured
by θ and Attachment to PD and TCD Surfaces
(Calculated from results presented in Figures 4.2 to 4.6
and Appendix Tables 6 to 9)

BACTERIAL SPECIES	PD		TCD	
	Duplicate Experiments		Duplicate Experiments	
	1	2	1	2
<i>Pseudomonas fluorescens</i>	-0.254	0.320	-0.089	-0.102
<i>Enterobacter cloacae</i>	-0.414	-0.352	-0.103	0.254
<i>Chromobacterium</i> sp	0.679 ^(a)	0.730 ^(b)	0.686 ^(a)	0.634 ^(b)
<i>Flexibacter</i> sp	0.558	0.838	0.707	0.826

The r for the *Chromobacterium* sp (a) represents 24 hr growth in media, (b) 48 hr growth.

Values of r greater than 0.707 differ significantly from 0 at 5% probability level.

experiments. Neither Pseudomonas fluorescens nor Enterobacter cloacae show any significant positive or negative correlation between cell surface hydrophobicity and their attachment to PD and TCD surfaces (Table 5.16).

The bacteria were each investigated for both θ_s and HIC (Table 5.17). There was no consistent relationship nor significant positive or negative correlation (the correlation co-efficient between θ_s and HIC for the bacteria was -0.468 values of r greater than 0.950 would be significant) was found between θ_s and the hydrophobic interactions of the four bacterial species.

5.6 DISCUSSION

5.6.1 The Role of Secondary Minimum and 1/K Thickness on Bacterial Attachment

There is evidence that secondary minimum and 1/K thickness may be important in the adhesion of both eukaryotic cells and bacteria. Red-blood cells were found to attach to a hexadecane interface at 10^{-3} M NaCl concentration but not at 2×10^{-4} M NaCl, i.e. larger 1/K. It was shown by interference microscopy that at the higher salt concentration the distance between the cell and the hexadecane surface was 100 nM which corresponded to the secondary minimum. At even higher NaCl concentrations (0.2M) there was apparently complete contact, possibly representing an interaction at the primary minimum (Gingell, 1978). Achromobacter R8 and Pseudomonas R3 were observed to increase the levels of reversible sorption to glass as 1/K decreased in size. At 1/K values of approximately 20 nM, there was total repulsion between Pseudomonas R3 and the glass surface, i.e. no attachment; at lower 1/K the bacteria were held a finite distance from the surface (Marshall et al. 1971b). The marine Pseudomonad was considered to be held at the secondary minimum since the kinetic energy the bacterium could produce by motility (5.45×10^{-9} μ J) was calculated to be insufficient to overcome the repulsion barrier

TABLE 5.17 The Relationship between θ_s and HIC Measurements for the Four Bacterial Species

Bacterial Species	$\theta_{H_2O}^a$ (degrees)	HIC (% cells retained in col)
<i>Pseudomonas fluorescens</i>	9.5	31.8
<i>Enterobacter cloacae</i>	11	81.8
<i>Chromobacterium</i> sp	17	25.4
<i>Flexibacter</i> sp	11	67.6

^a θ_{H_2O} measurements were made on cells prepared for HIC (i.e. 3 washes). Measurements were made as described in text.

between the primary and secondary minima.

However, the evidence that this effect is maintained in the irreversible sorption of bacteria is less clear. The results presented in this Chapter suggest that double-layer thickness is not a prime determinant of permanent bacterial attachment to surfaces. Indeed for the Flexibacter sp. smaller $1/K$ caused decreased attachment. The data indicates that electrolytes can either promote or inhibit bacterial adhesion and that the effect is variable both for the electrolyte type and concentration, and for the bacterial species involved.

Several workers found that cations do promote bacterial attachment. Streptococcus faecium increased attachment in NaCl and KCl concentrations up to 0.1M (Østavik, 1977), while Ga^{3+} and Al^{3+} were found to promote the attachment of oral streptococci to solid surfaces (Olsson et al. 1976). However, other evidence suggests effects other than $1/K$ thickness. Streptococcus mitior and Streptococcus salivarius showed maximum deposition on a rotating disc at ionic strengths between 0.03 and 0.1M. Above a concentration of 0.1M there was a reduction in attachment. A similar peak, in attachment of these species was found for a polystyrene surface, however, the maximum salt concentration was 0.05M after which there was a lowering in attachment for both the above species. S. salivarius was the least affected by ionic strength, but showed generally lower attachment levels, indicating the variation in response between species (Rutter & Abbott, 1978). Fletcher (1980a) found that Al^{3+} and La^{3+} inhibited the attachment of marine pseudomonad (NCMB 2021), the effect becoming more pronounced with increasing concentration.

The effect of electrolyte concentration on $1/K$ thickness may then not be of prime importance in permanent bacterial attachment to a surface. Different electrolytes and electrolyte concentrations may exert their influence on firm attachment either by a direct effect on the bacterium,

or by an effect on the cell surface components, e.g. by screening or cross-linking thence modifying interactions between the bacterial and solid surfaces. It is apparent from data in this Chapter that different ionic strength media affect the growth of the freshwater bacteria investigated, indicating a physiological effect on bacteria. Indeed certain cations, e.g. Na^+ and Mg^{2+} are known to enter into specific physiological activities. This physiological effect of electrolytes might result in changes in bacterial cell surface characteristics (Chapter 4) and thence changes in their attachment to solid surfaces.

Since bacteria have both negatively and positively charged inogenic groups on their surface it is possible for electrostatic interactions to develop between electrolytes and bacterial surface molecules. This will have a large effect on short range interactions between a bacterium and solid surface and therefore on their permanent adhesion. Electrolytes may even alter the stereochemistry of bacterial surfaces with a subsequent influence on bacterial attachment. S. salivarius was thought to alter the attachment with ionic strength either because of a change in charge density of its polymer coat or because of physical changes in the conformation of its polymer induced by the electrolytes (Rutter & Abbott, 1978). The acidic adhesive exopolysaccharide of a marine Pseudomonad was thought to interact directly with Al^{3+} and La^{3+} lowering bacterial attachment (Fletcher, 1980a), and an interaction with Ca^{2+} and Mg^{2+} was also suggested (Fletcher & Floodgate, 1973). Indeed, it is possible to visualize bacteria as biological ion exchangers (Costerton et al. 1978).

Thus although decreasing $1/K$ may increase levels of reversibly sorbed bacteria at a solid liquid interface, the effect may only serve to increase the probability of a bacterium approaching a surface and does not represent a predominant determinant of permanent bacterial attachment to a surface. DLVO theory is non-specific and long range and excludes

consideration of specific short range interactions, such as hydrogen bonding etc (Maroudas, 1975^a) which seem to be the basis of bacterial permanent attachment and will depend on the molecular structure and stereochemistry of each surface.

5.6.2 The Role of Surface Free Energies in Bacterial Attachment

The results presented in Section 4.5.2 show that only E. cloacae complies with the thermodynamic model of Neumann et al. (1979) described in Section 5.2. P. fluorescens increases in attachment to TCD before the liquid surface tension falls below the cell's surface tension. The attachment of the remaining two bacteria to PD and TCD surfaces was unaffected by changes in liquid surface tension. In general then, the thermodynamic model of Neumann et al. (1979) does not appear to predict the attachment of at least three of the bacteria investigated. Indeed, the effect DMSO had on the physiology of the bacteria, since it has a major influence on their growth, may be a significant factor in these interactions. DMSO may affect the cell surface in two ways; (1) by affecting cell physiology and therefore changing cell surface characteristics (Chapter 4) or (2) by entering into a direct interaction with the cell surface and thereby altering its properties.

Other workers, however, have found that attachment is consistent with the thermodynamic model. Neumann et al. (1979) showed that platelet and neutrophil adhesion to polymer surfaces could largely be predicted by the model. The attachment of Staphylococcus epidermidis increased to FEP-teflon as compared with the less hydrophobic cellulose acetate, when the surface tension of the suspending medium was higher than that of the cells in agreement with the model (Hogt et al. 1982). Bacterial attachment to PD and TCD surfaces in a selection of alcohols showed a minimum between 64 and 69 mNm⁻¹ liquid surface tensions, and Fletcher (1983) suggested this might be related to the point where $\gamma_{LV} = \gamma_{BV}$.

Since the effect was identical for both PD and TCD surfaces, attachment as predicted was independent of γ_{SV} . Attachment results have supported the significance of changes in the free energy of the system, i.e. ΔG or ΔF^{adh} in adhesion processes, for example, negative ΔG increased bacterial adhesion while positive ΔG decreased attachment of Serratia marcescens, S. epidermidis and S. aureus (Gerson & Scheer, 1980). A similar thermodynamic model to that described above was developed to predict the phagocytosis of bacteria by platelets (Absolom et al. 1982a), and experimentally the model was found to accurately describe bacteria/platelet interactions.

There are several problems, both experimental and theoretical, with applying predictions based on surface free energy interactions to bacterial attachment to solid surfaces. Experimental methods for evaluating the surface tension of liquids are simple and easy to apply. The measurement of solid surface tension is a more complicated problem and estimating bacterial surface tension becomes fraught with difficulties.

The use of contact angle measurements (θ_s) on lawns of cells presents major interpretative problems. For the empirically derived equation to be applicable the liquid should not chemically interact with the solid surface. Both the molecules of the cell surface and water molecules can enter into a range of interactions, e.g. H-bonding and such interactions may give a false indication of cell surface energies.

Both techniques for evaluating the surface energies of bacterial cells, i.e. γ_c (Zisman, 1964) and θ_s derived from an empirical formula (Neumann et al. 1974) present difficulties particularly because of the structurally and chemically complex nature of the bacterial cells. It is unknown what effects partial drying of a lawn of cells will have on the bacterial cell surface though they may well be considerable. The penetration of the bacterial films by test liquids will alter measurements and may vary with the test liquid. Pethica (1980) has pointed

out problems related to the conditions of rigidity, homogeneity and insolubility demanded for meaningful θ measurements which are not met by a bacterial system. Indeed, cellular components have been shown to transfer to the liquid (Baier cited in Pethica, 1980), which will alter liquid surface tension, and therefore θ . Thus problems of measuring the surface free energies of cells are considerable.

In applying thermodynamic models to cellular adhesion the assumption is made that the influence of electrical charges can be neglected. Neumann et al. (1979) found that neutrophil adhesion occurred at γ_{LV} of $6.9 \mu\text{J}/\text{cm}^2$ (the proposed neutrophil surface tension). They suggested, since adhesion could not be ascribed to van der Waals attraction that electrostatic interactions may have been implicated, particularly as the interactions occurred in the polar medium of water. By changing the ionic strength of the medium they could change levels of attachment, indicating an electrical charge interaction. It is undoubtedly the case that the four species investigated in this Chapter respond to changes in ionic strength (Tables 5.1-5.4) and therefore may not comply with the requirement for valid application of Neumann's equation of state, that electrical charges can be neglected. Another indication of the charged nature of bacterial surfaces was shown by the species variable but often considerable interaction with EIC columns (Tables 4.7-4.10). Thus the probability is indeed that electrostatic interactions are involved in the attachment of these four bacteria and that electrostatic interactions cannot be ignored.

Further, in applying surface free energies to describe interactions between surfaces and phases, it should be known if the interactive groups of the phases are complementary, since to interact surfaces must exhibit the same type of forces. Thus, although surface free energy of a substratum may describe its bonding capacity, another surface with different bonding capacities may not be able to interact. Bacterial surface free

energy estimates do not describe localized cell wall features, and indeed as surfaces approach there may be induced changes in the molecular geometry which is unmeasurable.

Thus the use of surface energies to predict bacterial attachment has theoretical difficulties. Indeed, the contact angle measurements of lawns of bacterial cells, and therefore indirectly surface energies of cells, is a major problem in any prediction of thermodynamic interaction. This may be underlined by the results presented in this Chapter. Although θ_s measurements on cells may be to some extent misleading, if we assume they are valid, only Enterobacter cloacae conforms to thermodynamic predictions of attachment, indicating that electrical charge may play a significant role in the adhesion of the other three bacteria to solid surfaces causing the divergence from the thermodynamic model.

5.6.3 The Role of Hydrophobic Interactions On Bacterial Attachment

It has long been known that certain apparently hydrophobic bacteria, such as acid fast bacteria, would spontaneously pass into the oil phase of an oil/water mix (Mudd & Mudd, 1924). The attachment of a marine pseudomonad was found to be higher to hydrophobic substrata than hydrophilic high energy surfaces, suggesting a role for hydrophobic bonding (Fletcher & Loeb, 1979). A similar preference for attachment to hydrophobic surfaces was found for some freshwater bacteria (Pringle & Fletcher, 1983b; Marshall and Cruickshank (1973) found that two bacterial species, Flexibacter aurantiacus CW7 and Hyphomicrobium vulgare ZV380 were perpendicularly orientated at interfaces, air/water, oil/water and solid/water. They found no evidence that positive ionogenic groups were unevenly distributed over the cells surface causing the 'end on' orientation at a negatively charged interface. Since Tween-80 a neutral surfactant, prevented adhesion they suggested orientation and subsequent attachment was due to hydrophobic groups at the cell poles

which were rejected from the water phase. Other bacteria have been shown to orientate themselves 'end on' to an interface (Zvyagintsev, 1959; Sieburth, 1975). The Flexibacter sp. investigated in this Chapter also showed a perpendicular alignment at an oil/water interface (Chapter 7).

Staphylococcus epidermidis was found (Hogt et al. 1982) to have a relatively hydrophobic surface, which was determined by investigating its attraction to xylene, and to attach better to hydrophobic FEP-teflon than to the more hydrophilic cellulose acetate. If the bacterial surface was treated so it became more hydrophilic, e.g. with aqueous phenol extraction, then bacterial attachment to both surfaces decreased. However, other cell surface characteristics may have been changed. The encapsulated more hydrophilic Staphylococcus saprophyticus showed lower attachment to both surfaces than S. epidermidis (Hogt et al. 1982). A similar relationship between increased affinity to a hydrocarbon, i.e. increased hydrophobicity, and increased adherence to polystyrene surfaces was found for a variety of bacteria, e.g. Acinetobacter calcoaceticus Rag-1 (ATCC 31012) and Serratia marcescens (Rosenberg, 1981).

Solid surfaces such as teeth have been found to have a high proportion of apparently hydrophobic bacteria. Weiss et al. (1982), using phase partition between hydrocarbon and water to estimate hydrophobicity, found 72% of isolates from teeth were hydrophobic. The hydrophobicity of oral streptococci was proposed as playing a role in their colonization of the oral cavity (Olsson & Westergren, 1972). The interaction between bacteria and tissue cells has often been explained in terms of bacterial hydrophobicity. Enteropathogenic Escherichia coli was thought to adsorb to surfaces by hydrophobic interactions (Smyth et al. 1978). Similarly, bacterial hydrophobicity was found to play a role in the attachment of Acinetobacter calcoaceticus RAG-1 to human epithelial cells (Rosenberg et al. 1981). However, the picture is not always so clear. Proteus

mirabilis S1959 showed no surface hydrophobicity but attached well to epithelial cells, indicating that hydrophobicity did not play a dominant role in attachment (Rosenberg et al 1982). Several workers have found that the more hydrophobic a bacterium, the more likely it is to be phagocytized, another form of adsorption interaction probably related to changes in the free energy of a system (Section 5.2 and 5.6.2) rather than hydrophobic bond formation (Magnusson et al. 1977; van Oss & Gillmann, 1972; Absolom et al. 1982a and b).

Still other attachment phenomena have been described in terms of bacterial hydrophobicity. The accumulation of bacteria at a liquid/air interface has been correlated with bacterial hydrophobicity and explained in terms of possible hydrophobic interactions. Sub-surface bacteria were found to have a far wider range of hydrophobic, and hydrophilicity interactions than those at the air/liquid interface (Dahlbäck et al. 1981).

The results presented in this Chapter for the attachment of four bacterial freshwater isolates to a hydrophobic PD surface and more hydrophilic TCD surface indicate that hydrophobic bonds probably were not dominant in the attachment interactions. Bacterial attachment did not necessarily increase with bacterial hydrophobicity. The only positive correlations between bacterial hydrophobicity and attachment were found for the Chromobacterium sp. and the Flexibacter sp when their hydrophobicity was estimated by θ_s measurements. As pointed out above the interpretation of θ_s measurements is difficult because of both experimental and theoretical problems. There was no positive correlation between bacterial hydrophobicity, measured by the more reliable HIC method and attachment to either PD or TCD surfaces.

Thus the mechanism of attachment of these four bacterial species to PD and TCD surfaces does not seem to be dominated by hydrophobic bonding phenomena. Given the range of interactions that a solid surface and a

bacterial surface could enter into (Chapter 1, Table 1.1), there is an inherent danger in trying to attribute adhesion to one particular physico-chemical factor. The picture becomes even more complicated if microbial physiological activity influences attachment as it may, particularly with the Flexibacter sp (Chapter 4 and 7).

However, even though there is no direct relationship between cell hydrophobicity and attachment the possibility of an influence by hydrophobic bonding on bacterial attachment should not be ignored. Marshall and Cruickshank (1973) found that the addition of the non-ionic detergent Tween-80 inhibited the adhesion of Flexibacter aurantiacus CW7 and Hyphomicrobium vulgare ZV380 to interfaces. Similar observations were made in this investigation for P. fluorescens, E. cloacae, the Chromobacterium sp. and the Flexibacter sp. for an anionic detergent, RBS, and Tween-80. Though the extent of the effect varied with the substratum and the bacterium. Similarly, the effect of various ionic strengths on attachment (Section 5.4.1) varied with substratum and bacteria. Desorption experiments indicated that the bacterial species investigated in this Chapter have their attachment to PD and TCD disrupted to some extent by detergents. However, the extent of detachment varies with the surface, the bacterial species, and the surfactant, underlining the variation in the nature of the attachment interactions.

In general, attachment to the hydrophobic (PD) surface is disrupted to a greater extent than that to TCD, indicating a greater involvement of hydrophobic bonding with this surface, as would be expected. However, the attachment of Flexibacter sp to either surface is unchanged by Tween-80. No detachment occurred for any of the species with changes in the pH or electrolyte composition of the suspending medium. It may be that electrostatic interactions between surfaces are fairly stable, though it is interesting that the anionic detergent RBS did cause significant

bacterial detachment which varied with species and substratum.

The desorption of bacteria has been found by other workers to follow similar trends. A marine Pseudomonas sp. Ma 8 remained firmly attached in water, 0.1N HCl and buffer solutions, and salt solutions were found to stabilize the film. However, detergents such as SLS and Triton X-100 removed the film, though cell lysis played a role in this removal (Corpe, 1974b). The effect of various surfactants, e.g. Tween-80, SLS, on the desorption of Escherichia coli, Flavobacterium oceanosedimentum and Aeromonas proteolytica from sediments varied with species and detergent (Scheraga et al. 1979) and pH over a range from 2 to 10 also showed variable effects. Ørstavik, (1977) found that the adsorption of Streptococcus faecium to glass was reversed by Tween-80 but not by water, various salts and urea. This indicates again that hydrophobic bonding is involved in some attachment interactions.

The evidence in Chapter 4 (Tables 4.7-4.10) describes considerable changes in bacterial cell surface properties with growth rate. Both hydrophobic and electrostatic differences in cell surfaces may be reflected in changes in levels of bacterial attachment, since there is no positive correlation between just the one factor, hydrophobicity and attachment. Other workers have found no direct correlation between overall surface charge and bacterial attachment. By using oral streptococci of different surface charges, Olsson et al. (1976) found no relationship between attachment and bacterial charge. Similarly, various strains of Streptococci mutans and Streptococci sanguis showed no clear correlations between zeta potentials and deposition at a solid/liquid interface (Abbott et al. 1980). However, modification of the surface charge of Mycoplasma pneumoniae did alter attachment levels of this bacterium to glass (Feldner et al. 1983). This again points to the variation between the determinants of attachment with species and surface.

Hydrophobic bonding apparently plays a role in bacterial attachment mechanisms. However, there is a danger in considering this interaction as being the major physico-chemical component of bacterial attachment. The evidence presented above suggests it may be only one component in a complex range of interactions, its importance varying with the cell surface and environmental factors. Feldner et al. (1983) reached the conclusion that both hydrophobic and electrostatic forces were involved in the attachment of M. pneumoniae to glass and presented the mechanism as a complex multi-factorial process. It is interesting that even oil drops suspended in water can be charged (Douglas & Shaw, 1958) allowing the possibility for charge interactions even in this apparently 'hydrophobic' situation. Interactions between lipid surface films and bacteria were found to involve steric effects, charge and hydrophobic interactions (Kjelleberg & Stenström, 1980).

Hydrophobic bonding can be visualized as having three distinct levels of importance in bacterial attachment;

- 1) hydrophobic bonding as the dominant force in attachment,
- 2) hydrophobic bonding as an insignificant part of bacterial attachment mechanisms,
- 3) hydrophobic bonding of equal importance to other short range forces in attachment.

The relative importance of the hydrophobic effect may well vary with the attachment interaction.

The relationship between the various types of bonding phenomena may not be just a balance of forces but a complementation of different types of interactions. Doyle et al. (1982) have recently proposed a hypothesis for the mechanism of adherence of Streptococcus sanguis to hydroxylapatite. They suggest a form of positive cooperativity in which the formation of one bond energetically favours subsequent

bond formation. Hydrophobic bonds may stabilize adjacent ionic or hydrogen bonds. Thus hydrophobic disrupting agents, e.g. Tween-80 should be more effective in disrupting adhesion than ionic or H-bond disrupting agents, e.g. salt solutions, because hydrophobic stabilizing would to some extent protect electrostatic or H-bonds from disrupting agents. This was found for the four freshwater bacteria investigated above (Tables 5.9-5.13). The model (Doyle et al., 1982) indicates that it will be virtually impossible to isolate any one component as dominant in attachment. Additional hydrophobic sites adjacent to ionic groups on the cell surface will tend to increase adherence. This underlines the importance of the composition of the outer membrane in attachment. The particular mosaic of the charge or hydrophobic groups at cell surfaces may be of major significance in bacterial attachment, and indeed may be altered by the approach to a substratum.

Still other types of interactions, e.g. steric effects, ionic bridging phenomena etc. could be involved in attachment. The role of water itself, although implied in hydrophobic bonding, has not been considered (Chapter 8). Given the range of possible phenomena involved in bacterial attachment to solid surfaces in aquatic environments (Chapter 1) and given the variabilities in both the bacteria (Chapter 4), and solid surfaces (Chapter 6) it is likely that attachment involves many overlapping phenomena. The physico-chemical adsorption of bacteria onto solid surfaces may be a multivariant interaction and depend on the bonding compatibility between the two surfaces.

5.7 SUMMARY

1) Long range forces, e.g. diffuse electrostatic double-layer thickness, and short range forces, e.g. chemical bonding are physico-chemical phenomena involved in the reversible and irreversible attachment of bacteria to solid surfaces, respectively.

2) The attachment of bacteria, as negatively charged colloidal sized particles, may be governed by the DLVO theory and the size of the diffuse electrical double-layer ($1/K$). The balance of long range attractive forces, i.e. London van der Waals forces and repulsive forces in electrostatic interactions between particles varies with electrolyte concentration and valency. The smaller $1/K$ the closer the bacteria can approach to a solid surface via the secondary minimum. Although reversible bacterial adsorption phenomena may be explicable in terms of long range forces and the size of $1/K$ (which decreases with increased electrolyte concentration and valency), irreversible attachment cannot be so explained (Tables 5.1-5.4). The effect of long range forces may be to increase the potential of bacteria entering into permanent attachment with a surface, by increasing the number of bacteria in the vicinity of the surface. However, specific short range bonding interactions, e.g. ionic, hydrophobic, dipole-dipole, between a bacterium and a surface are the final determinants of levels of attachment.

3) Short range forces can be described in terms of surface energies and interfacial energies, i.e. surface and interfacial bonding capacities. Neumann et al. (1979) developed a simple thermodynamic model for bacterial attachment to solid surfaces. They proposed that bacterial attachment will increase with increasing surface tension by the solid substratum if the surface tension of the liquid is lower than that of the cells. If the surface tension of the liquid is larger than that of the cells the reverse is the case. Although representing a useful guide, there are several limitations to this approach. Estimates of cell surface tension are made by measuring liquid contact angles on lawns of cells. This may be unreliable both because of practical difficulties e.g. changes in the cell membrane by drying, or seepage of substances from the cell into the liquid and also because of interpretive difficulties, e.g. the liquid

such as water may be involved in interactions with the cells, e.g. H-bonding. Also methods of lowering the surface tension of the surrounding medium, e.g. by increasing DMSO concentration, may directly affect the bacteria. The theoretical problems in applying thermodynamic models to bacterial adhesion reside in the fact that to be valid the influence of electrostatic charges must be insignificant, the reverse is indicated by results in this Chapter. Also it is assumed that the bonding capacities of each surface are compatible, this may not be the case. Only one species of the four investigated in this Chapter complied with the thermodynamic model, which may be of limited use because of the problems described above.

4) There was no positive correlation between bacterial cell surface hydrophobicity and bacterial attachment to either PD or TCD surfaces. However, detachment experiments indicated that hydrophobic bonds may have been involved in the attachment interactions of some of the organisms particularly P. fluorescens and the Chromobacterium sp especially to the hydrophobic PD surface.

5) It is proposed that attachment of these freshwater bacteria was a multi-factorial interaction phenomenon, with both hydrophobic and charge interactions involved. The relative importance of each interaction varies with the bacterial surface chemical composition, geometry and group mosaics, with the solid substratum, and with environmental conditions e.g. cation concentration.

CHAPTER SIX

THE INFLUENCE OF NUTRIENTS AND CONDITIONING FILMS ON BACTERIAL
ATTACHMENT TO SURFACES6.1 AIMS

To determine the effect of complex and simple nutrients on the adhesion of freshwater bacteria to PD and TCD surfaces.

6.2 INTRODUCTION

In natural aquatic environments all surfaces acquire conditioning films since high molecular weight compounds will spontaneously adsorb to solid surfaces (Baier, 1980a, b). Such conditioning films will change the physico-chemical characteristics of the solid surface, e.g. the solid surface tension and the bonding capacity of the surface (Chapter 5). Indeed, conditioning films have been shown to influence levels of bacterial attachment, though much of the work has been with pure protein conditioning films (Meadows, 1971; Fletcher, 1976). It is possible that not only are solid substrata associated with molecular conditioning films but that bacterial surfaces will also acquire these layers which, in turn, will have their effect on bacterial attachment to solid surfaces. Further, high molecular weight compounds will alter the liquid surface tension of the suspending medium and this may also influence bacterial attachment levels (Chapter 5).

To determine the effects of the nutrient conditioning films on the attachment of Pseudomonas fluorescens, E. cloacae, the Chromobacterium sp, and the Flexibacter sp, to PD and TCD surfaces, these bacteria were attached in the presence of PYE nutrients. The role of variations in cell surface characteristics caused by changes in growth conditions, and the influence of increased physiological activity, due to the presence of nutrients on bacterial attachment, was investigated.

There is some indication that low molecular weight compounds such as

glucose can affect levels of bacterial attachment (Marshall et al. 1971b). A range of carbohydrates, metabolizable and non-metabolizable, and a range of glucose concentrations were used to investigate the influence of low molecular weight compounds in the attachment of these four bacteria, particularly P. fluorescens. Again changes in the growth conditions of the bacteria prior to their attachment were used to investigate the role of cell surface characteristics and physiological factors on the interaction between bacterial attachment and the low molecular weight compounds.

6.3 MATERIALS AND METHODS

6.3.1 Organisms

The four freshwater bacterial species, Pseudomonas fluorescens, (H_2), Enterobacter cloacae (H_{12}), a Chromobacterium sp (H_{31}) and a Flexibacter sp. (H_{38}), described in Chapter 4, were used in the investigations.

6.3.2 Culture Conditions and Inoculation Procedure

(a) Batch Culture.

Pseudomonas fluorescens was cultured in glucose minimal medium (Section 2.3.4). One ml aliquots of a P. fluorescens-glucose minimal medium stock culture were inoculated into 100 ml of fresh media. The cultures were incubated at 15°C on a rotary incubator at 150 rpm for 24 hrs before sampling.

(b) Continuous Culture.

i) PYE medium

All four bacterial isolates were grown in pure continuous culture in PYE media. The inoculation procedure, growth conditions and dilution rates, i.e. 0.05, 0.1, 0.15 and 0.2 h^{-1} , were identical to those previously described (Section 4.3.2(b)(i)).

ii) Minimal media

The bacteria were also grown in pure continuous culture ($D = 0.025\text{ h}^{-1}$) in three separate minimal media conditions (Section 4.3.2(b)(ii)).

- 1) carbon (glucose)-limited
- 2) nitrogen-limited
- 3) carbon-and nitrogen-sufficient

The media, inoculation, and growth rate of the cultures were identical to those outlined in Section 4.3.2(b)(ii).

6.3.3 Attachment Assays

The minimal media batch cultures and 100 ml samples of both the minimal media continuous cultures and PYE medium continuous cultures were centrifuged at 10,960 av.g (Beckman model J-21B centrifuge). The cells were then washed once in 0.01M HEPES buffer (pH 7.4), before one of four different attachment assays was initiated, as follows;

i) Bacteria grown in PYE continuous culture, at one of several growth rates used, were resuspended in (a) 0.01M HEPES buffer (pH 7.4), (b) 0.05% (w/v) peptone, 0.035% (w/v) yeast extract in 0.01M HEPES buffer (pH 7.4), or (c) 0.1% (w/v) peptone, 0.07% (w/v) yeast extract in 0.01M HEPES buffer (pH 7.4), to an optical density of 0.1 at 540 nm in a colorimeter (Corning Colorimeter 252). The standard incubation, washing and staining procedures (Section 2.3.4) were then followed for duplicate PD and TCD surfaces for each attachment condition for both 5 min and 60 min incubation periods.

Results were expressed both as $A_{590}(\times 10^{-3})$ of the attached cells on the surface and an index of attachment (I_a). I_a was calculated as the ratio of the $A_{590}(\times 10^{-3})$ of the test substratum against the relevant control substratum, i.e. PD test/PD control, TCD test/TCD control. I_a values of 1 were recorded for treatments where 95% confidence limits of the mean overlapped with the controls, any value greater than or less than 1 was considered to represent an effect.

ii) Bacteria grown in minimal medium in continuous culture were resuspended in (1) 0.01M HEPES buffer (pH 7.4), (2) 0.5 mg/ml glucose

in 0.01M HEPES buffer (pH 7.4), or (3) 2 mg/ml glucose in 0.01M HEPES buffer (pH 7.4), to an optical density of 0.1 at 540 nm in a colorimeter. After 60 min incubation at 15°C duplicate plates of PD and TCD were washed and stained using the standard assay procedure (Section 2.3.4).

iii) P. fluorescens grown in glucose minimal medium batch cultures was resuspended in 0.01M HEPES buffer (pH 7.4) to an OD of 0.1 (540 nm) in a colorimeter. One of the two procedures was then followed:

(a) Pretreatment experiments: Duplicate PD and TCD surfaces were exposed to 100 μ l of one of four carbohydrates, glucose, mannitol, and inositol, to a final concentration (after addition of the bacterial suspension) of 1% (w/v). To the 'pre-treated' surfaces, 5 ml aliquots of bacterial cells suspension were added.

(b) Post-treatment experiments: 5 ml aliquots of bacterial suspension were placed in duplicate PD and TCD surfaces. To these was added 100 μ l quantities of one of the four carbohydrates listed above, to the final concentration of 1% (w/v).

After 60 min incubation at 15°C the standard attachment assay procedure was adopted (Section 2.3.4).

6.3.4 Contact Angle Measurements on Clean and Treated PD and TCD Surfaces

PD and TCD surfaces were exposed to 5 ml aliquots of 0.05% (w/v) peptone, 0.035% (w/v) yeast extract in 0.01M HEPES buffer (pH 7.4); and 0.1% (w/v) peptone, 0.07% yeast extract in 0.01M HEPES buffer (pH 7.4). The surfaces were then incubated at 15°C for 60 min before being washed 3 times with 0.01M HEPES solution. The treated surfaces were then allowed to air-dry for a minimum of 2 hrs at room temperature.

The advancing water contact angle (θ_{H_2O}) of clean and treated PD and TCD surfaces was then measured, using the procedure described in Section 5.3.6.

6.3.5 Liquid Surface Tension Measurements

The liquid surface tension of 0.01M HEPES (pH 7.4); 0.05% (w/v) peptone, 0.035% (w/v) yeast extract in 0.01M HEPES (pH 7.4); 0.1% (w/v) peptone 0.07% (w/v) yeast extract in 0.01M HEPES (pH 7.4); 0.5 mg/ml glucose and 2 mg/ml glucose were measured using the technique previously described in Section 5.3.7.

6.4 RESULTS

6.4.1 The Effect of Bacterial Growth Rate and the Presence of Complex Nutrients on Bacterial Attachment

Two concentrations of PYE medium were used to determine the effect the presence of complex nutrients had on the attachment of P. fluorescens, E. cloacae, a Chromobacterium sp, and a Flexibacter sp, to PD and TCD surfaces. From a preliminary survey of Tables 6.1 to 6.16 the extent of variation in the effect with different surface, organism and bacterial growth rate can be seen to be large.

The PD I_a and TCD I_a values indicated that either an increase or decrease in attachment resulted from the presence of nutrients (Tables 6.1 to 6.16). This effect varied not only with the solid substratum, but also with the concentration of nutrients present. The 5 min and 60 min I_a values for both surfaces did not necessarily show the same effect, e.g. the PD I_a 5 min for P. fluorescens ($D = 0.15 \text{ h}^{-1}$) attached in 0.1% peptone and 0.07% yeast extract showed an increase in attachment over the control, but there was a reduction for PD I_a 60 min (Table 6.3). The interactions changed with the growth rate of the organisms, e.g. the Chromobacterium sp. attached for 60 min in 0.1% peptone and 0.07% yeast extract showed an increase in attachment to PD at a growth rate of 0.05 h^{-1} but a decrease at $D = 0.15 \text{ h}^{-1}$ (Tables 6.9 and 6.11). The treatments also affected the rate of increase in attachment from 5 min attachment to that at 60 min e.g. the Chromobacterium sp. ($D = 0.05 \text{ h}^{-1}$) showed a far larger rate of

TABLE 6.1 The Effect of Nutrients on the Attachment of Pseudomonas fluorescens to PD and TCD after growth in continuous culture at a dilution rate of 0.05 h^{-1}

Treatment	PD	5 min attachment				60 min attachment			
		TCD	PD I_a^b	TCD I_a^b		PD	TCD	PD I_a	TCD I_a
Control	12(± 2) ^a	21(± 3)	1	1		25(± 3)	27(± 3)	1	1
0.05% peptone									
0.035% yeast extract	16(± 0.8)	26(± 2)	1.3	1		44(± 8)	66(± 8)	1.76	2.4
0.1% peptone									
0.07% yeast extract	14(± 2)	23(± 0.8)	1	1		22(± 4)	50(± 8)	1	1.9

^aParenthetical values are 95% confidence limits of the mean ($n = 8$)

^b I_a = Index of attachment (see page 160).

TABLE 6.2 The Effect of Nutrients on the Attachment of Pseudomonas fluorescens to PD and TCD after growth in continuous culture at a dilution rate of 0.1 h^{-1}

Treatment	PD	5 min attachment				60 min attachment			
		TCD	PD I_a	TCD I_a		PD	TCD	PD I_a	TCD I_a
Control	6(± 3) ^a	14(± 0.8)	1	1		30(± 7)	25(± 3)	1	1
0.05% peptone									
0.035% yeast extract	16(± 3)	20(± 2)	2.7	1.4		26(± 5)	59(± 8)	1	2.36
0.1% peptone									
0.07% yeast extract	12(± 3)	26(± 2)	1	1.86		20(± 4)	62(± 4)	1	2.48

^aParenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.3 The Effect of Nutrients on the Attachment of Pseudomonas fluorescens to PD and TCD after growth in continuous culture at a dilution rate of 0.15 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	$9(^{+0.8})^a$	$19(^{+2})$	1	1	$20(^{+2})$	$32(^{+3})$	1	1
0.05% peptone								
0.035% yeast extract	$11(^{+0.8})$	$24(^{+3})$	1.22	1	$18(^{+3})$	$37(^{+3})$	1	1
0.1% peptone								
0.07% yeast extract	$15(^{+2})^a$	$27(^{+2})$	1.67	1.42	$11(^{+2})$	$30(^{+3})$	0.55	1

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.4 The Effect of Nutrients on the Attachment of Pseudomonas fluorescens to PD and TCD after growth in continuous culture at a dilution rate of 0.2 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	6(± 2) ^a	13(± 2)	1	1	32(± 4)	21(± 3)	1	1
0.05% peptone 0.035% yeast extract	6(± 0.8)	15(± 2)	1	1	6(± 2)	25(± 4)	0.18	1
0.1% peptone 0.07% yeast extract	6(± 0.8)	20(± 3)	1	1.54	5(± 2)	19(± 3)	0.16	1

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.5 The Effect of Nutrients on the Attachment of Enterobacter cloacae to PD and TCD after growth in continuous culture at a dilution rate of 0.05 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	8(± 2) ^a	19(± 3)	1	1	31(± 3)	43(± 4)	1	1
0.05% peptone 0.035% yeast extract	10(± 4)	16(± 3)	1	1	61(± 6)	26(± 3)	1.97	0.6
0.1% peptone 0.07% yeast extract	10(± 2)	18(± 3)	1	1	38(± 4)	32(± 3)	1	0.74

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.6 The Effect of Nutrients on the Attachment of Enterobacter
cloacae to PD and TCD after growth in continuous culture at a
dilution rate of 0.1 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	7(± 0.8) ^a	17(± 0.8)	1	1	13(± 2)	31(± 5)	1	1
0.05% peptone 0.035% yeast extract	8(± 2)	17(± 2)	1	1	37(± 3)	27(± 2)	2.06	1
0.1% peptone 0.07% yeast extract	7(± 0.8)	17(± 0.8)	1	1	44(± 3)	27(± 4)	2.44	1

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 6.7 The Effect of Nutrients on the Attachment of Enterobacter
cloacae to PD and TCD after growth in continuous culture at a
dilution rate of 0.15 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	3(± 3) ^a	17(± 3)	1	1	5(± 3)	24(± 3)	1	1
0.05% peptone 0.035% yeast extract	6(± 2)	13(± 3)	1	1	22(± 2)	12(± 2)	4.4	0.5
0.1% peptone 0.07% yeast extract	2(± 0.8)	9(± 0.8)	1	0.53	27(± 2)	13(± 0.8)	5.4	0.54

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

TABLE 6.8 The Effect of Nutrients on the Attachment of Enterobacter
cloacae to PD and TCD after growth in continuous culture at a
dilution rate of 0.2 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	5(± 0.8) ^a	17(± 2)	1	1	8(± 3)	22(± 4)	1	1
0.05% peptone 0.035% yeast extract	3(± 2)	14(± 2)	1	1	12(± 3)	13(± 2)	1	0.59
0.1% peptone 0.07% yeast extract	5(± 2)	13(± 3)	1	1	17(± 2)	14(± 2)	2.13	0.64

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.9 The Effect of Nutrients on the Attachment of the Chromobacterium
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.05 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	14(± 3) ^a	16(± 3)	1	1	34(± 6)	13(± 4)	1	1
0.05% peptone 0.035% yeast extract	23(± 2)	13(± 3)	1.64	1	76(± 8)	10(± 0.8)	2.2	1
0.1% peptone 0.07% yeast extract	17(± 2)	9(± 2)	1	0.56	86(± 8)	5(± 3)	2.53	0.38

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.10 The Effect of Nutrients on the Attachment of the Chromobacterium
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.1 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	14(± 0.8) ^a	25(± 2)	1	1	37(± 2)	39(± 3)	1	1
0.05% peptone								
0.035% yeast extract	15(± 3)	25(± 3)	1	1	70(± 7)	32(± 5)	1.89	1
0.1% peptone								
0.07% yeast extract	12(± 2)	18(± 3)	1	0.72	54(± 4)	21(± 5)	1.46	0.54

^aParenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.11 The Effect of Nutrients on the Attachment of the Chromobacterium
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.15 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	21(± 4) ^a	23(± 4)	1	1	70(± 3)	59(± 5)	1	1
0.05% peptone								
0.035% yeast extract	21(± 5)	27(± 2)	1	1	83(± 8)	76(± 3)	1.18	1.29
0.1% peptone								
0.07% yeast extract	9(± 3)	19(± 2)	0.43	1	38(± 3)	24(± 5)	0.54	0.41

^aParenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.12 The Effect of Nutrients on the Attachment of the Chromobacterium
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.2 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	27(± 3) ^a	29(± 0.8)	1	1	84(± 8)	56(± 4)	1	1
0.05% peptone 0.035% yeast extract	20(± 3)	25(± 2)	0.74	0.86	63(± 8)	35(± 4)	0.75	1.21
0.1% peptone 0.07% yeast extract	9(± 3)	19(± 3)	0.33	0.66	50(± 3)	25(± 3)	0.6	0.86

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.13 The Effect of Nutrients on the Attachment of the Flexibacter
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.05 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	9(± 2) ^a	7(± 3)	1	1	15(± 3)	7(± 3)	1	1
0.05% peptone 0.035% yeast extract	6(± 2)	3(± 3)	1	1	15(± 2)	12(± 3)	1	1
0.1% peptone 0.07% yeast extract	7(± 3)	2(± 2)	1	1	12(± 3)	13(± 3)	1	1

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.14 The Effect of Nutrients on the Attachment of the Flexibacter
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.1 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	13(± 2) ^a	13(± 2)	1	1	51(± 5)	76(± 7)	1	1
0.05% peptone 0.035% yeast extract	11(± 2)	11(± 3)	1	1	28(± 2)	36(± 0.8)	0.55	0.47
0.1% peptone 0.07% yeast extract	10(± 3)	14(± 0.8)	1	1	22(± 2)	33(± 3)	0.43	0.46

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.15 The Effect of Nutrients on the Attachment of the Flexibacter
sp to PD and TCD after growth in continuous culture at a
dilution rate of 0.15 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	10(± 0.8) ^a	17(± 3)	1	1	101(± 17)	90(± 15)	1	1
0.05% peptone 0.035% yeast extract	3(± 2)	13(± 3)	0.3	1	44(± 2)	48(± 3)	0.44	0.53
0.1% peptone 0.07% yeast extract	5(± 0.8)	18(± 3)	0.5	1	24(± 4)	36(± 3)	0.24	0.4

^a Parenthetical values are 95% confidence limits of the mean ($n = 8$)

TABLE 6.16 The Effect of Nutrients on the Attachment of the Flexibacter
 sp to PD and TCD after growth in continuous culture at a
 dilution rate of 0.2 h^{-1}

Treatment	5 min attachment				60 min attachment			
	PD	TCD	PD I_a	TCD I_a	PD	TCD	PD I_a	TCD I_a
Control	32(± 3) ^a	25(± 3)	1	1	108(± 23)	111(± 10)	1	.1
0.05% peptone								
0.035% yeast extract	7(± 0.8)	16(± 0.8)	0.22	0.64	79(± 8)	68(± 4)	0.73	0.61
0.1% peptone								
0.07% yeast extract	7(± 0.8)	15(± 3)	0.22	0.6	64(± 0)	55(± 3)	0.59	0.49

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

attachment in 0.1% peptone, 0.07% yeast extract than it did in the buffer control (Table 6.9).

The only species to show a consistent relationship between attachment levels and nutrient concentration despite changes in growth rate, was the Flexibacter sp. The presence of nutrients at all but the lowest growth rate, where there was no apparent effect, caused decrease in attachment to both PD and TCD surfaces (Tables 6.13 to 6.16).

The results presented in Chapter 4 showed either an increase in attachment or no effect on bacterial attachment with increase in growth rate. However, the results in this Chapter of attachment in the presence of nutrients, show increases, decreases or no affect with increasing D , suggesting growth rate is not a prime factor in determining attachment here. Some other factors may have predominated in the adhesive interaction. These were probably the physico-chemical influences of adsorbed nutrients on the surfaces, indicated by θ_{H_2O} measurements on clean and nutrient surfaces differing (Table 6.17), and/or by modification of the solid-liquid surface tensions (Tables 6.17 and 6.18).

6.4.2 The Effect of Growth Conditions on the Influence of Glucose Concentrations on Bacterial Attachment

The four bacterial species were grown in carbon-limited, nitrogen-limited and carbon/nitrogen-sufficient conditions in continuous culture ($D = 0.025 \text{ h}^{-1}$). Each species was then attached in glucose concentrations which represented carbon-limited (0.5 mg/ml) and carbon-sufficient (2 mg/ml) conditions.

The levels of bacterial attachment varied with species, growth condition, the glucose concentration of the suspending medium and substratum. In the majority of experiments there was no difference between control surfaces and those attachments carried out in the presence of glucose (Fig. 6.1 to 6.4). However, P. fluorescens and the Chromo-

TABLE 6.17 Water Contact Angle (θ_{H_2O}) of PD and TCD surfaces treated with Peptone, Yeast Extract Solutions

Surface/ Treatment	$\theta_{H_2O}^\circ$	Solid Surface Tension ($\mu J/cm^2$)
PD - clean	90(± 0.2) ^a	2.85
PD - A	72.5(± 2)	3.90
PD - B	80(± 2)	3.47
TCD - Clean	59(± 1)	4.72
TCD - A	18.5(± 2)	6.83
TCD - B	13(± 2)	7.02

^a Parenthetical values represent 95% confidence limits of the mean (n = 10)

Treatment A - 0.05% (w/v) peptone, 0.035% (w/v) yeast extract

Treatment B - 0.1% (w/v) peptone, 0.07% (w/v) yeast extract

TABLE 6.18 Liquid Surface Tension Measurements of Peptone, Yeast Extract Solutions

Solution	Liquid Surface Tension ($\mu J/cm^2$)
0.05% peptone 0.035% yeast extract	6.6
0.1% peptone 0.07% yeast extract	6.4
HEPES (0.01M)	7.0
0.5mg/ml glucose	7.0
2 mg/ml glucose	6.8

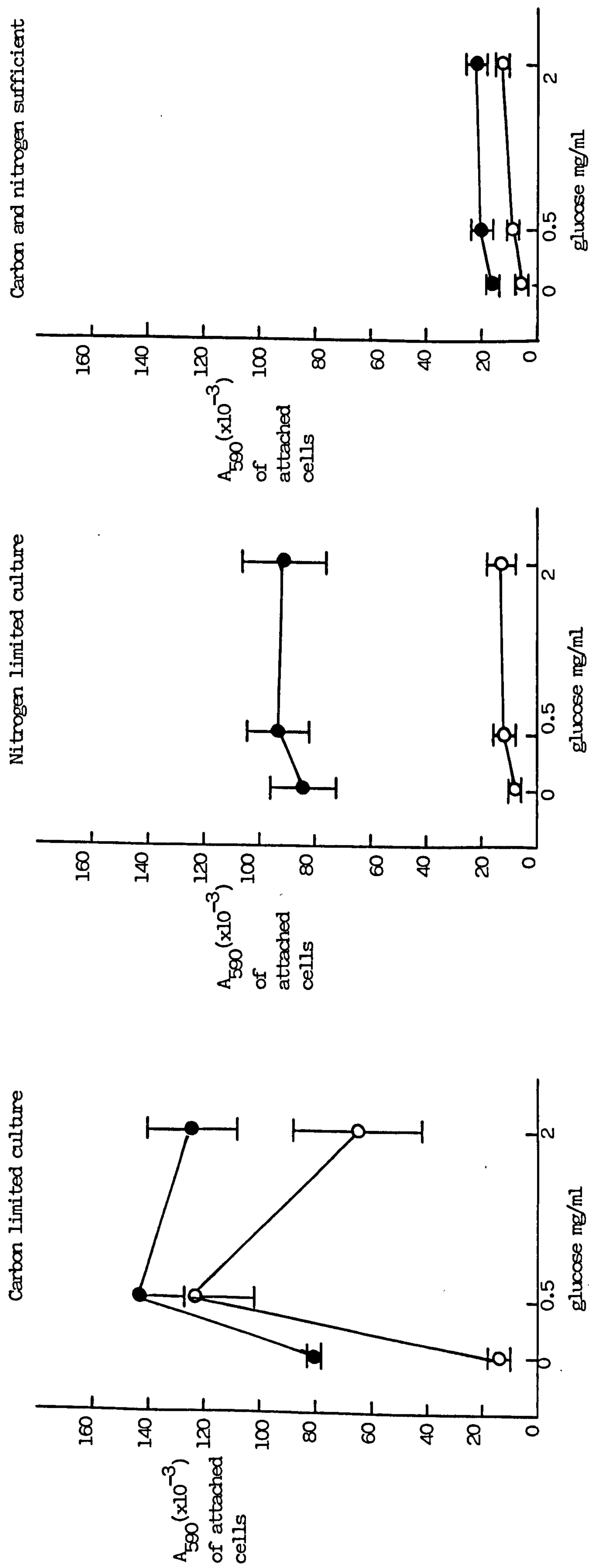


Figure 6.1 The effect of glucose concentration on the attachment of *Pseudomonas fluorescens* after growth in continuous culture (D = 0.025h⁻¹) in glucose limited, nitrogen limited and glucose/nitrogen sufficient conditions

(O), cells attached to PD surface; (●), cells attached to TCD surface;

The bars represent the 95% confidence limits of the mean (n = 8)

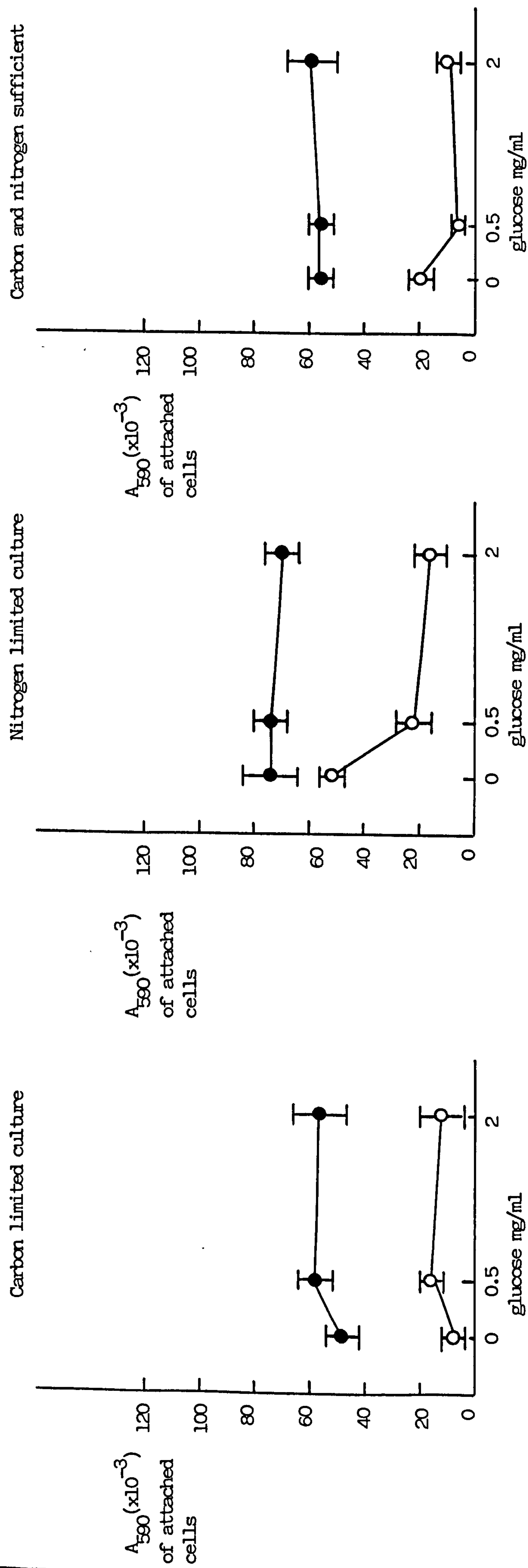


Figure 6.2 The effect of glucose concentration on the attachment of *Enterobacter cloacae* after growth in continuous culture ($D = 0.025 \text{ h}^{-1}$) in glucose limited, nitrogen limited and glucose/nitrogen sufficient conditions.

(O), cells attached to PD surface; (●), cells attached to TCD surface.

The bars represent the 95% confidence limits of the mean (n = 8)

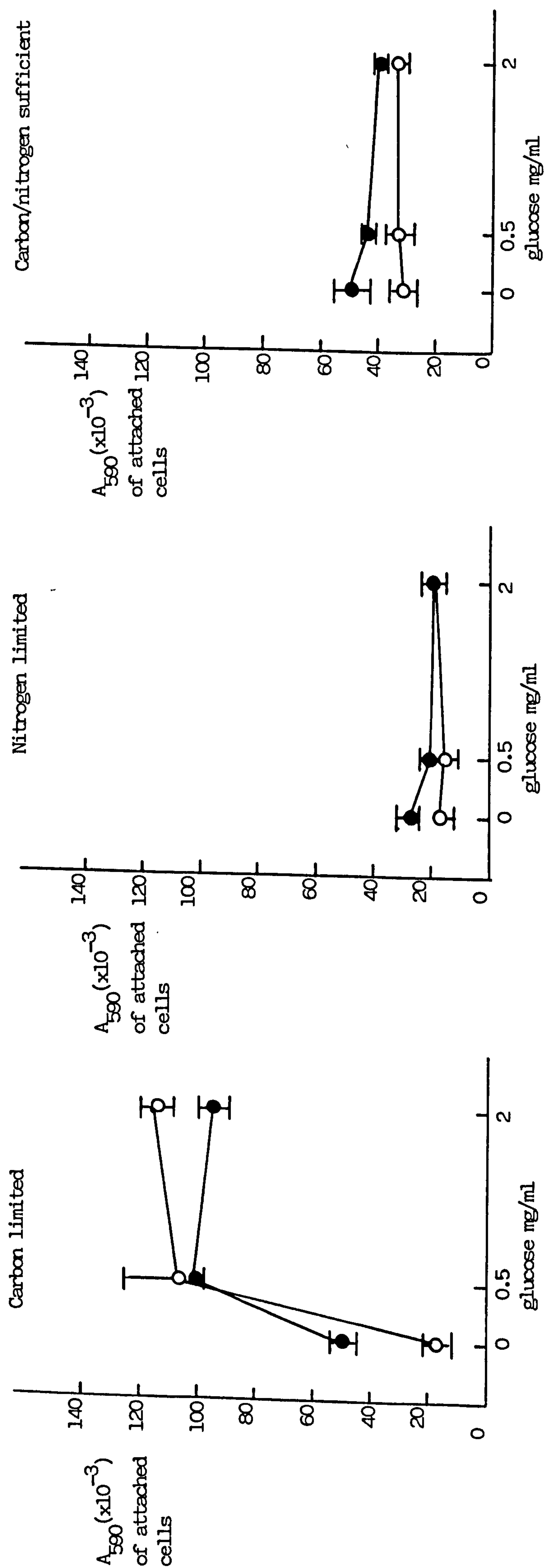


Figure 6.3 The effect of glucose concentration on the attachment of the *Chromobacterium* sp after growth in continuous culture ($D = 0.025 \text{ h}^{-1}$) in glucose limited, nitrogen limited and glucose/nitrogen sufficient conditions.

(O), cells attached to PD surface; (●), cells attached to TCD surface

The bars represent the 95% confidence limits of the mean ($n = 8$)

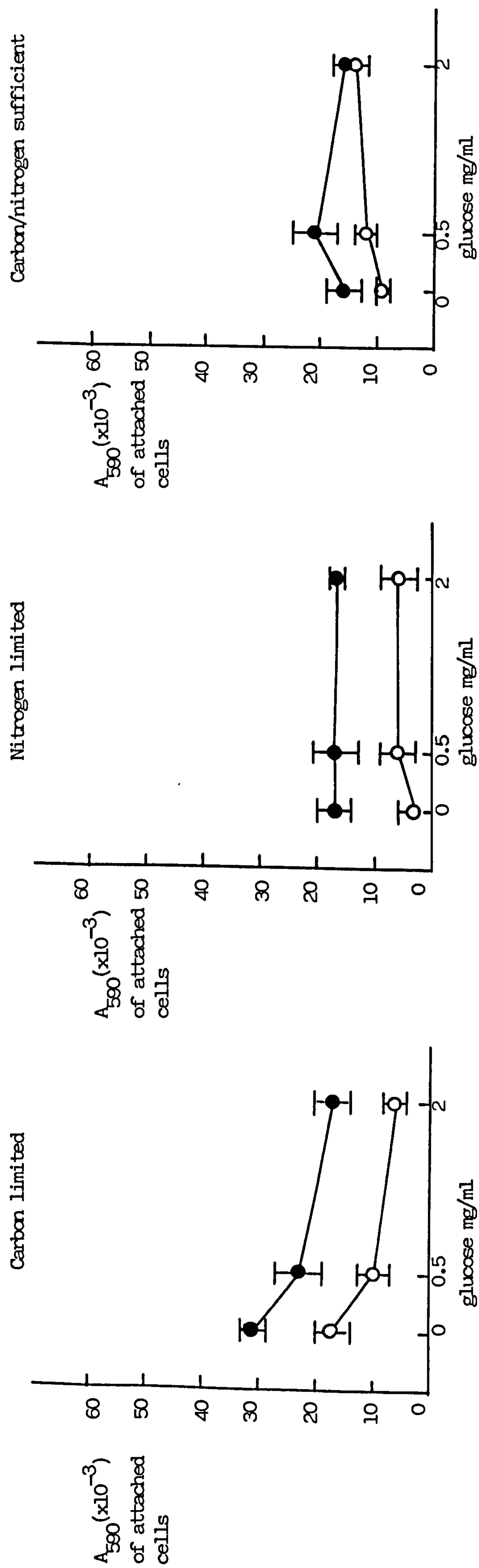


Figure 6.4 The effect of glucose concentration on the attachment of the *Flexibacter* sp after growth in continuous culture ($D = 0.025h^{-1}$) in glucose limited, nitrogen limited and glucose/nitrogen sufficient conditions (O), cells attached to PD surface; (●), cells attached to TCD surface. The bars represent the 95% confidence limits of the mean ($n = 8$)

bacterium sp. showed an increase of attachment to both surfaces in the presence of glucose after growth in carbon-limited conditions (Fig. 6.1 and 6.3) while the Flexibacter sp. had a decrease in attachment with these conditions (Fig. 6.4). The effect of glucose was different for the two surfaces for E. cloacae after growth in nitrogen-limited and carbon/nitrogen-sufficient conditions in that the attachment to TCD was unaffected by glucose concentration, however that to PD dropped (Fig. 6.2).

Growth rates were maintained at $D = 0.025 \text{ h}^{-1}$ for all the growth conditions; however, different glucose/nitrogen ratios have been shown to alter cell surface characteristics (Chapter 4). Changes in glucose concentrations of the suspending medium altered the liquid surface tension of the solution (Table 6.18).

6.4.3 The Effect of Pre-Treatment and Post-Treatment of substrata with Carbohydrates on the Attachment of PSEUDOMONAS FLUORESCENS

The effect of metabolizable (glucose and mannitol) and non-metabolizable (maltose and inositol) carbohydrates on the attachment of P. fluorescens was investigated (Fig. 6.5 and 6.6). The levels of bacterial attachment did not seem to be dominated by the potential use of the carbohydrate as a substrate, but rather by physico-chemical phenomena. If the carbohydrates were added to the PD surface before the cell suspension was added, i.e. pre-treatment, then levels of attachment increased over that of the control. However, there was no such affect if the carbohydrates were added after the cell suspension, i.e. post-treatment. In general, a similar interaction was found for the TCD surface although two of the carbohydrates, mannitol and maltose, had no effect on levels of attachment with pre-treatment.

(Appendix Tables 17 to 22 contain repeat experiments for the carbohydrate experiments).

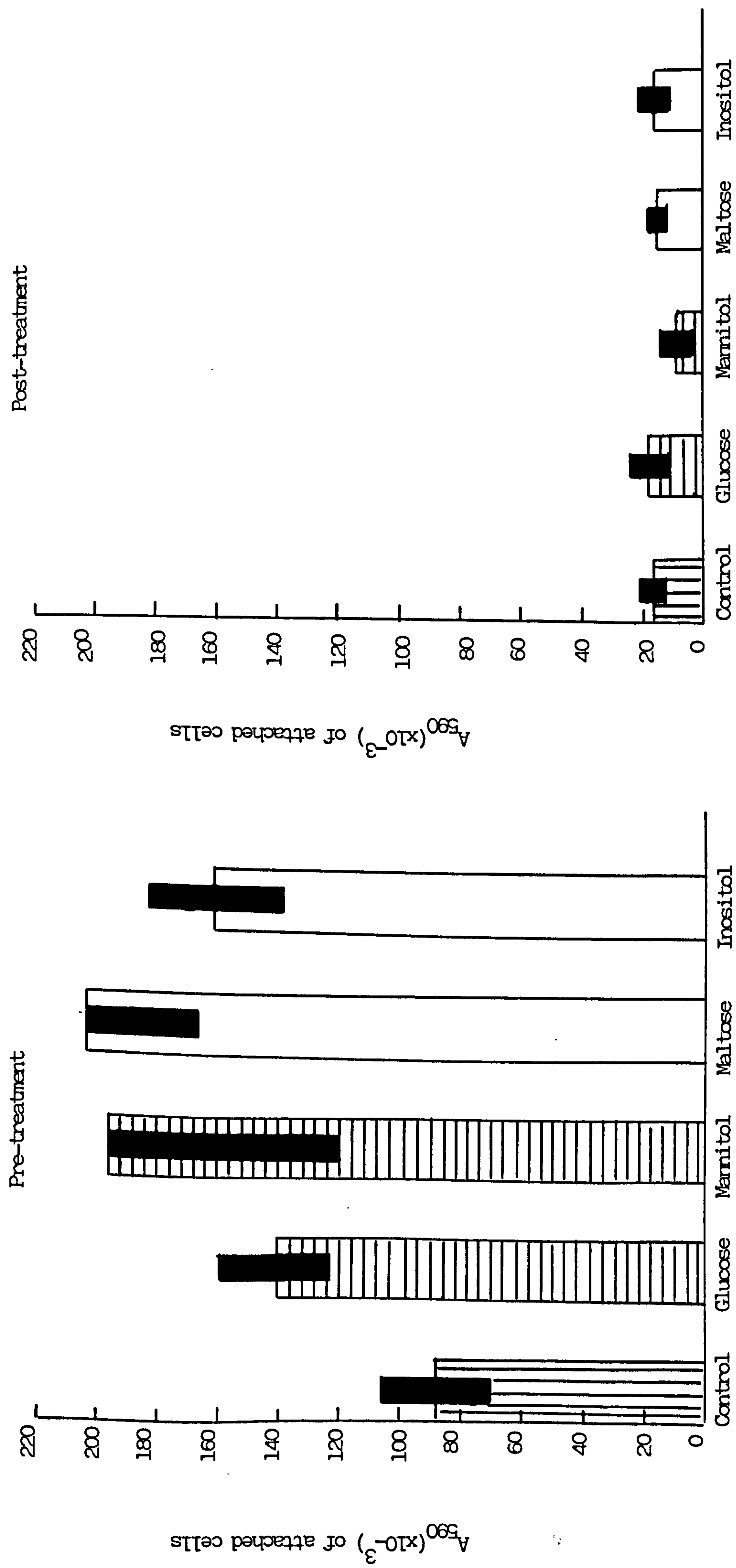


Figure 6.5 The effect of pre-treatment and post-treatment of PD surface with carbohydrates on the attachment of *Pseudomonas fluorescens*. (■), control; (▨), metabolizable carbohydrate, (□), non-metabolizable carbohydrates; the bars represent the 95% confidence limits of the mean (n = 8)

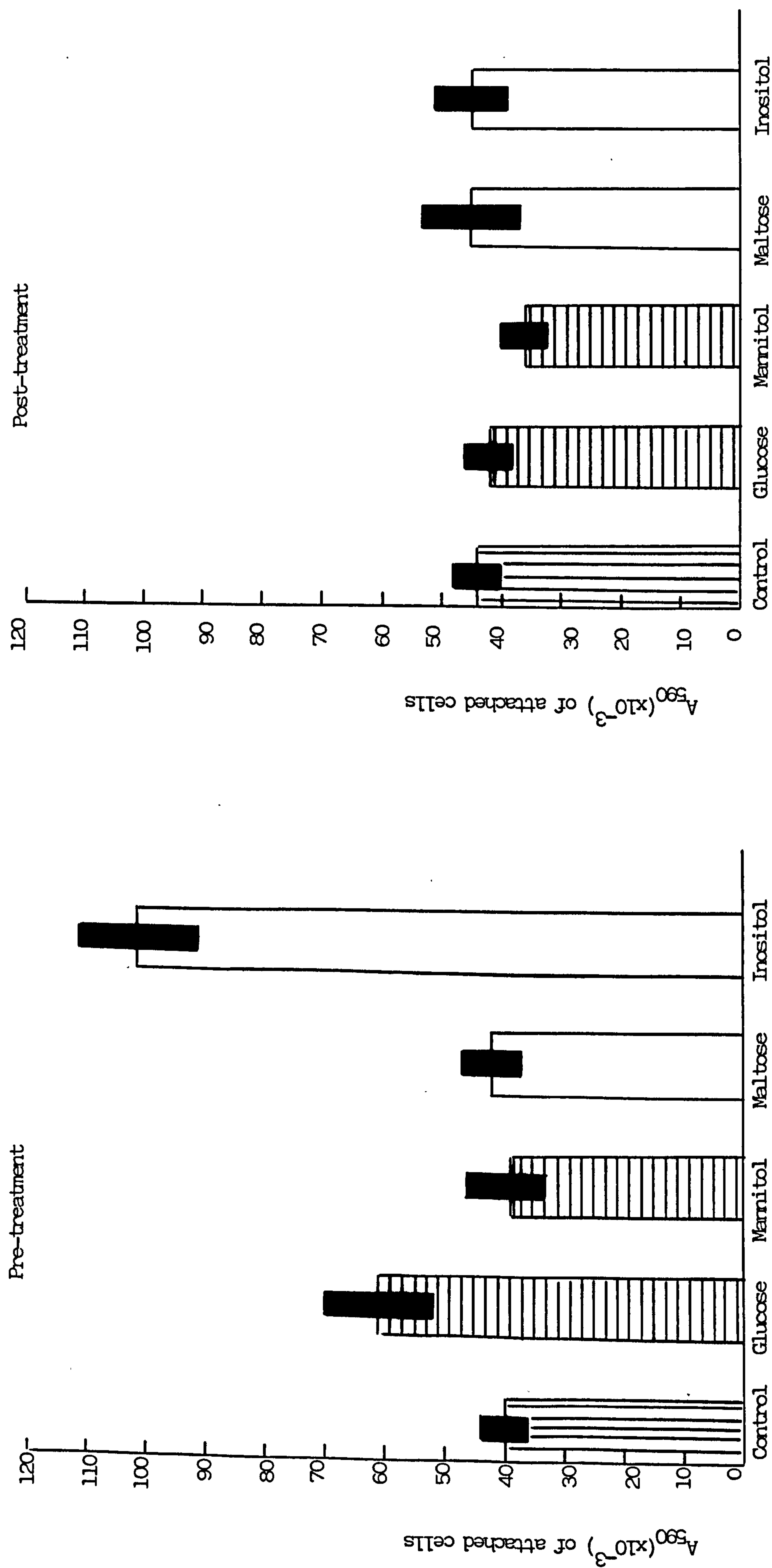


Figure 6.6 The effect of pre-treatment and post-treatment of TCD surface with carbohydrates on the attachment of *Pseudomonas fluorescens* (□), control; (▨), metabolizable carbohydrates; (■), non-metabolizable carbohydrates. The bars represent the 95% confidence limits of the mean (n = 8)

6.5 DISCUSSION

6.5.1 The Effect of Nutrients on the Solid Substratum, Liquid Phase and on Bacterial Attachment

The adsorption and subsequent firm attachment of bacteria to solid surfaces was clearly influenced by the presence of complex nutrients in the liquid bulk phase. The results presented above indicate a diverse interaction varying with bacterial characteristics, the solid surface and the concentration of molecules in the liquid phase.

The influence of added molecular species to the liquid phase is clear. The interactivity of the liquid phase will change, since there are new molecular types with different interaction potentials, which in turn will result in thermodynamic parameters, such as the liquid surface tension, altering. This is clearly demonstrated by the liquid surface tension measurements on the two concentrations of PYE solutions (Table 6.18).

Organic molecules, both macromolecules and smaller molecules, tend to accumulate spontaneously at a solid-liquid interface. This is favoured, since the accumulation of an organic layer at an interface will result in a reduction of interfacial free energy. This may constitute an important driving force in the formation of a conditioning layer, however, entropy effects may also be involved (Dexter, 1977) as will hydration effects. Smaller organic molecules will tend to set up an adsorption equilibrium involving both molecular adsorption to, and desorption from, the surface. The macromolecules, on the other hand, will normally not desorb since they are held to the solid surface by large numbers of anchoring, adsorption sites. It is statistically unlikely that all the anchoring sites will desorb in unison (Kipling, 1965). The resultant layer of organic molecules at a surface may be referred to as a conditioning film. It should be noted, however, that a conditioning film may not be complete over the entire solid surface, but that gaps may expose the original surface.

6.5.2 The Nature and Formation of Conditioning Layers

The formation of a conditioning layer by the PYE concentrations on both PD and TCD surfaces was clearly indicated by the somewhat crude method of drying the adsorbed conditioning layers on the surfaces and measuring θ_{H_2O} . (Section 6.3.4 and 6.4.1). In all probability both the reversible adsorption of small molecules and the largely irreversible adsorption of macromolecules are involved in the development of these conditioning films since PYE consists of a mixture of peptides and many smaller molecules e.g. amino acids, carbohydrates.

The θ_{H_2O} measurements on PD and TCD showed that the surfaces were affected to different extents by the conditioning film. The θ_{H_2O} changes on TCD were greater than those of PD. Both varied with the concentration of the solutions. Using bubble contact angles to determine differences in interfacial tensions, Fletcher and Marshall (1982) also found that the conditioning film apparently differed between PD and TCD, and changed with the composition of the macromolecular solution, e.g. bovine serum albumin, bovine glycoprotein, and its concentration. They found that the conditioning film could be modified by the use of pronase. The extent of enzymatic degradation not only varied with the macromolecular species but also with the solid surface, suggesting that the configuration of proteins on the surface varied between PD and TCD surface.

The amount and rate of protein adsorption is dependent on the solid surface involved (Kim & Lee, 1975). Factors such as abilities of the solid and macromolecules to enter into hydrogen and electrostatic bonding, and hydrophobic interactions will influence molecular adsorption. Differences in the adsorption of macromolecules onto different substrata have been shown by several workers. MacRitchie (1972) found that greater levels of bovine serum albumin adsorbed onto Aerosil R-972, a colloidal silica with a hydrophobic surface, then onto Aerosil 200, a hydrophilic silica.

Hydrophobic Teflon-FEP (Fluorinated ethylene-propylene copolymer) was shown to adsorb fewer proteins than other less hydrophobic polymers, e.g. polydimethyl siloxane, and the protein was apparently less firmly held (Baszkin & Lyman, 1980). Differences in the adsorption characteristics of the PD and TCD surfaces may partly account for θ_{H_2O} differences of adsorbed films between the two surfaces described above. The difference in the effect of pronase treatment on proteins adsorbed to PD and TCD found by Fletcher and Marshall (1982) may also partly be explained by differences in extent of adsorption between surfaces.

The adsorption will not only vary with the solid surface, but also with the macromolecular species being adsorbed. Norde and Lyklema (1978) theorised that the affinity of proteins for negative surfaces will increase with increasing hydrophobicity of the macromolecule, with the number of positive groups located at its surface and also with the extent of the re-arrangement of the protein once adsorbed.

The structure and configuration of adsorbed macromolecules has often been shown to differ considerably from that assumed in aqueous solution. For example, the double helix of DNA was opened and unwound to a single stranded form at a solid/liquid interface (Numberg & Valenta, 1978). Not only did configurations differ between phases but molecular arrangements were different for weakly charged interfaces and strongly charged interfaces. At strongly charged interfaces the adsorption involved all the base residues, on weakly charged interfaces only the Adenine-Thyamine base pairs were involved in deconformation, i.e. dissociation and adsorption (Sequaris et al. 1978).

Different macromolecules will also react in different ways to solid surfaces; for example, some may be anchored by one end only while others may be flat on a surface, binding along their entire length, their configuration having been unfolded. Still others may be attached

at a few points and present coiled structures to the bulk liquid phase (Eirich, 1977). In general, high energy and high polarity substrata will adsorb macromolecules strongly and they will become flattened across the surface. Low energy non-polar surfaces will bond macromolecules less strongly although a thicker layer may develop (Baier, 1980b).

Thus the macromolecules themselves in adopting different adsorption configurations expose different surface characteristics to the bulk phase.

Norde and Lyklema (1978) proposed a model for protein adsorption suggesting that the adsorbed protein consists of 3 distinct regions:

- 1) the region nearest the solid phase contains positive groups ion-paired to negative groups on the solid surface,
- 2) the middle region, which is devoid of charged groups and
- 3) the region nearest the bulk liquid phase containing another band of charged groups.

The substratum surface will, then, assume the net surface charge of the outermost portion of the protein. Hence, the solid surface will assume the surface free energy of the outermost section of protein and, as a result, new bonding capacities and interaction capabilities. The role of the conditioning film in conferring a new surface free energy to the solid PD and TCD surfaces will also be reflected in θ_{H_2O} measurements (Table 6.17).

There are a large array of different macromolecules found in aquatic environments. The majority of these are of biological origin and include proteins, glycoproteins, proteoglycans and polysaccharides. As early as 1943, ZoBell showed that organic matter was concentrated by adsorption onto solid surfaces, and since then the nature and extent of the effect of conditioning layers has been investigated. In a number of different natural environments the development of conditioning films, as would be predicted from the results described above, has been shown to occur

instantaneously on a clean surface. The initial interactions of blood with a foreign surface involves the adsorption of a strongly adherent proteinaceous film. This occurs within 5 secs of contact (Baier & Dutton, 1969). In seawater (Baier, 1980b) there was rapid formation of a glyco-proteinaceous conditioning film, which increased in thickness with the length of exposure to the surrounding bulk phase. Other organic molecules became incorporated in the film with increased exposure times. Lipids were incorporated in the proteinaceous film in interactions with blood (Baier & Dutton, 1969), and in the oral cavity muco-polysaccharide was added to the developed glycoprotein film (Baier, 1974). The thickness of the film in aquatic environments possibly ranges from 10 to 20 nm (Baier, 1980b).

The macromolecular conditioning film will have a large influence on the characteristics of a solid surface. The solid substratum will no longer dictate the surface characteristics presented to the bulk phase, but the outermost conditioning layer will. Different particles, e.g. anion exchange resin, quartz, which exhibit a range of surface charges all tended to show a zero or moderately negative charge when immersed in seawater (Neihof & Loeb, 1974; Loeb & Niehof, 1975). This was clearly demonstrated to be the result of adsorption of organic molecules onto the particles, with the effect developing very rapidly. The surface charge of the solid is not the only characteristic to change, but the critical surface tension of the substratum changes with the accumulation of the conditioning layer (Baier, 1980b).

6.5.3 The Role of Conditioning Layers on Bacterial Attachment to Solid Surfaces

In natural environments, bacteria do not interact with clean surfaces, since they do not exist in natural circumstances, but rather with the organic films conditioning those surfaces. Thus conditioning films will

play a significant role in attachment interactions in freshwater and marine habitats. However, not only will solid substrata acquire conditioning films, but so too will microorganisms. Bacteria already present a complex array of macromolecules to the surrounding environment, the characteristics of which have been shown to change with growth rate and growth condition (Chapter 4). The potential for interactions with molecules in the surrounding bulk liquid phase is large and not only governed by physico-chemical factors, but also associations may result from bacterial physiological processes, e.g. active uptake systems. Since both cell surface characteristics and bacterial activity change with conditions, a conditioning film around the bacterium may well not only vary with species but also with growth conditions. Similarly, with the solid substratum the interaction may also vary with the organic molecules present in the surrounding liquid phase.

The presence of conditioning layers, either on the substratum or possibly associated with the bacterial surface has been shown to influence bacterial attachment both in vivo and in vitro. A selection of proteins were found to either inhibit or promote bacterial attachment (Meadows, 1971), and a marine pseudomonad's attachment to polystyrene was shown to be impaired by the presence of bovine serum albumin, gelatin, fibrinogen and pepsin (Fletcher, 1976). There was evidence that the bovine serum albumin not only conditioned the polystyrene but also the bacterial surface in the latter case. The attachment of Mycoplasma pneumoniae to glass surfaces, was reduced in the presence of protein and fatty acid-free bovine serum albumin and the effect was least intense in the lag phase of growth (Feldner et al 1983).

In natural environments as diverse as blood and seawater, Baier (1974) considers that the development of a conditioning film on solid substrata is a pre-requisite for adhesion to occur. He suggested a minimum adsorbed film thickness of 20 nm was

necessary for cell deposition, a threshold which would take 1-2 min to develop in nutrient-sufficient environments but possibly many hours in dilute environments. It has been found in several different environments that there is a range of substratum γ_c 's at which attachment numbers are minimum, i.e. 200 to 300 NN/cm² for bacteria in seawater (Dexter et al. 1975; Dexter, 1977). This range of γ_c 's has been termed the biocompatibility range. The dip in attachment in the biocompatibility range often takes a few hours to develop. Dexter (1977) found that in natural seawater the biocompatibility range did not start to appear until 5 hrs of substratum immersion and was not established until 24 hrs. This delay was considered to indicate that the biocompatibility range was not a direct result of substratum critical surface tension, but rather a result of the organic conditioning film. Baier (1974) suggested that configuration changes in adsorbed glycoproteins caused by denaturation of the molecules on the substratum enhanced subsequent attachment of cells. Within the biocompatibility range such changes were small and as a consequence bacterial attachment from natural seawater was minimal. Dexter (1977, 1979) later proposed that the biocompatibility range represented the solid critical surface tension which approaches the surface tension of the liquid. The system then behaves as if the interface were not present and interfacial energy is 0 (Chapter 5). This entropic stabilisation would tend to prevent adsorption. Not only would this influence bacterial attachment, but also the formation and nature of a conditioning layer.

The results presented in this Chapter suggest that conditioning films play a considerable role in bacterial attachment. The effect varies with the substratum and the concentration of the suspending medium. On the basis of the foregoing discussion such factors would be expected to alter bacterial attachment interactions. More interesting was the effect of the bacterial surface characteristics on the interaction. It was clearly

demonstrated in Chapter 4 (Tables 4.6 - 4.9) that cell surface characteristics change with growth rate, the extent of change depending on species (NB the data in Tables 4.6 to 4.9 can be related directly to Tables 6.1 and 6.16 since the experimental conditions were identical). Clearly, bacterial surface characteristics severely modify bacterial interactions with conditioned surfaces and undoubtedly the nature of their own 'conditioning film'.

Bacterial attachment was found to vary between 5 min attachment and 60 min attachment (Tables 6.1 to 6.16). Undoubtedly the conditioning film would be thicker at the longer attachment period, and the attachment of the bacteria themselves would, to some extent, modify the attachment interactions (Chapter 3). Although the organic film on the TCD, i.e. the more hydrophilic surface, might be more firmly held and the peptides more denatured than on the PD surface, the conditioning films on the surface resulted in inhibition or stimulation to bacterial attachment (Tables 6.1 to 6.16). The prime determinant again was apparently the nature of the bacterial surface. Interestingly, not only total attachment numbers but also the rate of attachment could be considerably altered by the presence of PYE. Thus initial levels of attachment were the same in buffer and PYE for certain organisms, but the presence of PYE produced subsequent rapid increases in attachment. This may indicate the type of attachment interactions occurring in the buffered solution and PYE solutions were different.

The fact that the conditioning films changed the surface free energy of the substrata in the systems described in this Chapter (Table 6.17) indicates that the apparent bonding capacities of the PD and TCD surfaces were changed. Indeed not only the number but also the types of available bonds would be altered. Thus the types of possible attachment interactions (Section 1.4) between the solid surfaces and the bacterial surfaces would change.

The conditioning layers may change the particular electrostatic mosaics on both the solid surface and bacterial surface or may influence the ionic environment at the surface (Brooks & Seamann, 1973) and in this way influence long-range interactions. Conditioning films can exert two further influences, those of polymer bridging and steric exclusion.

Polymer bridging can occur when two surfaces, one or both with polymeric films, approach since it becomes possible for segments of the same polymer molecule to attach to both surfaces, anchoring the two together. This interaction is favoured when the surfaces are not entirely covered by polymer; it becomes unlikely with saturated surface coverage (Lips & Jessup, 1979). The term steric exclusion comprises two features: the first is a geometric exclusion of interaction by rigid molecular structure, e.g. loops. The second is the tendency to maintain minimum free energy of the system, since entropy effects favour the maximum freedom and randomness of attached coiled polymers (Maroudas, 1973, 1975b). Steric exclusion by conditioning films will act against bacterial attachment.

Since PYE contains few peptide chains and no proteins it is difficult to judge the influence of polymer bridging and steric exclusion in PYE on bacterial attachment; they may be slight.

At short range, osmotic factors may also tend to cause repulsion in the presence of organic surface films (Lips & Jessup, 1979). A further important influence of conditioning films is due to changes in solution viscosity. The high viscosity of organic components might hinder bacterial adhesion, since it limits drainage from the interface (Rutter & Vincent, 1980).

A further, and possibly negative, influence on bacterial attachment by nutrients may be a competition for adsorption sites on the solid surface between the bacteria and organic molecules. The adsorption of the nutrients

molecules would render an adsorption site on the substratum inaccessible to attaching bacteria. If adsorption sites are limited the result may be to lower numbers of bacteria attaching to the surface.

6.5.4 The Effect of Nutrients on Bacterial Activity and Attachment

The presence of nutrients during bacterial attachment may well increase the bacteria's physiological activity which, in turn, may affect their attachment.

The data presented in this Chapter indicates that increased activity either by increasing growth rate, or by including nutrients in the suspending solution does not necessarily increase attachment. Thus the prime influence of nutrients in the attachment solution may not be physiological but rather physico-chemical.

The attachment of the Flexibacter sp. has already been suggested to increase with bacterial activity in buffered solutions (Chapter 4). This effect was muted in the presence of nutrients, which uniformly inhibited permanent attachment of this bacterium. The Flexibacter sp. a bacterium capable of a gliding motility, again, then differs from the other three non-gliding bacteria in its attachment. This bacterium may possess a different attachment system than the other three bacteria, since it seems to be disrupted by the presence of nutrients in the attachment suspension. This may be due to the effect of the conditioning layer on the substratum, the effect of the suspending medium itself on the Flexibacter sp, or possible viscosity changes affecting this species' attachment (Chapter 7).

6.5.5 The Role of Simple Organic Molecules on Bacterial Attachment

The influence of simple carbohydrates on bacterial attachment to solid surfaces involves a system of organic molecules which should not form a permanent association with the solid surface, an equilibrium being

set up between adsorption and desorption.

Equation 6.1: solid surface + carbohydrate $\xrightleftharpoons[K_2]{K_1}$ solid surface-carbohydrate complex

K_1 and K_2 represent the rate of adsorption and desorption respectively.

The rates will be influenced by the nature of the solid surface and the carbohydrate. A similar description can be made for the bacterial surface though there will be an added component in the adsorption interaction if the bacterium can assimilate the carbohydrate. In this case the bacterium may bind the carbohydrate at an uptake site and will act as a sink for the molecules. The presence of carbohydrate molecules in the aqueous phase will decrease the liquid surface tension, the effect becoming more pronounced with increasing organic concentration.

There was a wide variation in the effect of glucose concentrations on the attachment of P. fluorescens, E. cloacae, the Chromobacterium sp, and the Flexibacter sp, after growth in different carbon/nitrogen ratio media. The cultures were maintained at a constant growth rate of 0.025 h^{-1} . Changes in the growth conditions alone caused differences in levels of bacterial attachment to PD and TCD surfaces in buffered systems. This was probably due to differences in the cell surface characteristics of the bacterium (Chapter 4). The addition of different concentrations of glucose to the attachment solution further modified attachment levels variably with substratum and species and may be the result of physico-chemical interactions.

Marshall et al. (1971b) found that adsorption of Pseudomonas R3 to glass surfaces was stimulated by 2 mg/l glucose but was completely inhibited by the presence of 30 or 70 mg/l glucose in the attachment solution. These concentrations are far less than those used in the experiments described in this Chapter, which were found to be either inhibitory or stimulatory to attachment. Ellwood et al. (1982) refining

a suggestion of Marshall, put forward the proposal that high affinity uptake systems for glucose might recognize glucose molecules concentrated near the solid surface and 'dock' with them, thus adhering to the substratum. As glucose concentration increased (Marshall et al 1971b) so the uptake sites on the bacteria in the aqueous bulk phase become saturated and unable to 'dock' with molecules on the solid surface. Thus inhibition of attachment would occur at higher glucose concentrations. Further, Ellwood et al. (1982) observed an increase in bacterial diversity and numbers with an absence of extracellular polymer, when bacteria were attached in glucose-limiting growth conditions. They suggested that glucose limitation and the resultant more efficient bacterial glucose uptake system (Herbert & Kornberg, 1976) increased the bacteria's potential for docking.

A problem with this interpretation is that the glucose uptake system is not located on the outer surface of the bacteria, but rather on the plasma membrane, and therefore may not be available for the docking attachment interaction visualized above. The results presented in this Chapter, further, suggest that a docking system may not determine the bacterial attachment in these systems. The concentrations of glucose in the suspending medium would saturate potential uptake sites many times over, and although there was an increase in attachment for two species in carbon-limited conditions there was not for the other two. Indeed attachment in carbon-sufficient conditions could be high. However, the carbohydrate molecules need not only react with specific sites on the bacterial surface but rather interact with the bacterium in a physico-chemical manner. The bonding capacities and type of potential bonding by both the substratum and bacterium would change with the association of glucose at their surfaces. Also, since glucose concentrated at an interface would be in equilibrium with that in the bulk phase or libel

to uptake by the bacterium, unless the bacteria once docked at a glucose molecule stabilized the interaction by bonding in other ways, it is doubtful if permanent attachment would result. Thus bacterial surface characteristics even in this model of attachment would be of a predominant importance.

Interestingly it was possible to elicit different attachment interactions with both PD and TCD, by pre and post-treatment with different carbohydrates. The generally stimulatory effect on the attachment of P. fluorescens by pre-treatment may be the result of the association between the carbohydrates and the solid surface being different from that resulting from post-treatment. The equilibrium balance of adhesion and desorption by the organic molecules may have varied between the two resulting in a physico-chemical effect on bacterial attachment. Both metabolizable and non-metabolizable carbohydrates had the same effect, though TCD changed its interaction with the particular carbohydrate.

In this study both complex and simple organic molecular systems can then affect bacterial attachment, probably by altering physico-chemical interactions rather than by changing levels of bacterial activity and thence altering attachment.

6.6 SUMMARY

1) The presence of complex organic molecules in the liquid bulk phase causes the instantaneous formation of a molecular conditioning film on a solid surface. The nature of the film is influenced by the solid substratum e.g the more hydrophilic the surface the more compact and denatured the macromolecular layer, and varies with the organic molecules and concentration.

2) Once conditioned, the solid surface no longer has a direct influence on bacterial attachment, but an indirect one since the conditioning film

represents the surface that a bacterium will encounter. Thus the charge and surface free energy of the conditioning layer determine bacterial attachment, although there is the possibility of incomplete surface coverage.

3) The bacterial surface itself may develop a conditioning layer, which will vary with the original cell surface characteristics

4) The influence of conditioning films on attachment will be exerted through a number of different phenomena.

a) differences in the bonding capacities as compared with the 'clean' surface, either promoting, inhibiting or not affecting attachment

b) osmotic factors inhibiting attachment

c) steric exclusion phenomena inhibiting attachment

d) polymer bridging enhancing attachment

e) viscosity effects tending to inhibit attachment

f) a competition for adsorption sites on the solid surfaces between the bacteria and the organic molecules in solution.

The interplay and resultant balance of these forces will determine the levels of attachment

5) Thus the solid substratum, the nature and concentration of the organic molecules and the cell surface characteristics of the bacteria all interact to determine levels of adhesion

6) The presence of simple carbohydrate molecules also influenced bacterial adhesion, the effect changing with bacterial surface characteristics and growth conditions, and the solid substratum. It is probable that the effect is physico-chemical, resulting from changes in bonding capacity and potential bonding types, with the development of equilibrium associations of the carbohydrate with the bacteria and solid substrata. These associations would vary with the nature of the solid surface and the bacterial surface and hence affect attachment.

CHAPTER SEVEN

A COMPARISON IN THE ATTACHMENT CHARACTERISTICS BETWEEN A GLIDING BACTERIUM (FLEXIBACTER SP) AND THREE NON-GLIDING BACTERIA7.1 AIMS

To contrast and compare the attachment of a gliding bacterium, Flexibacter sp, with three non-gliding bacteria, and to investigate the temporary and permanent adhesive mechanisms of the Flexibacter sp.

7.2 INTRODUCTION

The majority of bacteria undergoing irreversible attachment (Section 1.4) to solid surfaces are permanently attached to that surface. They are apparently no longer motile and are often embedded in a polymeric matrix. The bacteria are effectively immobilized by the substratum. Three of the species described in this, and previous Chapters, fall into this category, P. fluorescens, E. cloacae and the Chromobacterium sp. It may be in natural environmental conditions that this type of permanent association dominates bacterial/solid surface interactions.

However, certain bacterial species are able to glide across a solid surface, e.g. Cytophaga, and, therefore, are not only capable of permanent attachment to the substratum. Gliding motility implies a different type of adhesive interaction between the solid and bacterial surfaces, combining bacterial attachment with the potential for mobility over the surface. Marshall (1980) termed this temporary adhesion. Gliding motility is characterized by movement in the direction of the long axis of the cell, which is fully reversible (Henrichson, 1972). Such motility occurs at solid/liquid, liquid/oil and liquid/air interfaces. Clearly, the interactions involved in temporary adhesion

are very different from those of permanent adhesion. To underline this point cells of Flexibacter BH3 have been observed to half 'peel-off' from a substratum so that half the cell protruded into the water phase (Duxbury et al. 1980). The Flexibacter sp. investigated in this Chapter and previous Chapters was able to glide across a substratum (Chapter 2), and thus to undergo temporary adhesion. In natural environments the importance of gliding bacteria on surfaces may be underestimated, since normal sampling techniques, e.g. washing a surface to remove loosely attached bacteria, might remove all but permanently attached bacteria (Marshall, 1980).

Permanent and temporary adhesion must involve different types of interactions with the solid surface. As a result the attachment, and the effect of environmental factors on attachment, of bacteria capable of these two different adhesive types may be different from those of permanently attached bacteria. Further, the ability of an organism to demonstrate gliding motility on a solid surface indicates perhaps a higher degree of adaptation to colonization of interfaces than those bacteria which permanently attach. This in itself suggests that gliding bacteria may react differently to a solid/liquid interface and may show specialized characteristics in their attraction and subsequent association with the interface. Thus the gliding bacterium and the three non-gliding bacteria described above may react very differently in their irreversible attachment to solid surfaces.

Similarities and differences in the permanent irreversible attachment characteristics of the Flexibacter sp, and the three non-gliding bacteria to PD and TCD were therefore investigated, particularly with respect to environmental factors such as pH and temperature and with respect to growth phase in batch culture prior to the attachment. The role of different cell surface components in

attachment to solid surfaces was investigated for the four species by using degradative agents. While nutrient conditions favouring the gliding motility of the Flexibacter sp, were also examined.

7.3 MATERIALS AND METHODS

7.3.1 Organisms

Pseudomonas fluorescens (H₂), Enterobacter cloacae (H₁₂), a Chromobacterium sp, (H₃₁) and a Flexibacter sp (H₃₈) were investigated. Three of the four isolates, P. fluorescens, E. cloacae and Chromobacterium sp, attached permanently to a solid surface, while the fourth, Flexibacter sp, was capable both of a gliding motility across a solid surface and of permanent adhesion (Chapter 4).

7.3.2 Culture Conditions and Inoculation Procedure for the Attachment and Detachment Experiments

Pure batch cultures of the four bacterial species were grown in PYE medium broth (Section 2.3.2). The medium was inoculated with 1 ml aliquots of PYE medium broth stock cultures, and incubated at 15°C on a rotary incubator at 150 rpm for 16 hrs (unless otherwise stated) before harvesting.

7.3.3 Growth and Motility of the Bacterial Species at Temperatures between 4°C and 45°C

Pure bacterial cultures were grown for 24 hrs at 15°C on PYE medium agar (Section 2.3.2). Samples were then stab inoculated into capped sterile 12 mm tubes containing a semi-solid medium agar. The medium consisted of the Hugh Leifson's oxidative test medium (Hugh & Leifson, 1953)(Chapter 2, Section 2.3.3(10)). Unsealed (the media were not covered with liquid paraffin) duplicate tubes were incubated for each bacterial species at a range of temperatures, 4°C, 15°C, 20°C, 25°C, 37°C and 45°C. After 5 days incubation the amount of growth and spread, i.e. motility, from the original inoculum was estimated.

7.3.4 Growth of the Bacterial Species over a Range of pH

Bacterial cultures were grown in PYE medium broth (Section 2.3.2) for 24 hrs at 15°C. 100 µl aliquots of these pure cultures were then inoculated into 5 ml tubes containing 0.1% (w/v) peptone; 0.07% yeast extract and a 0.01M buffer mixture adjusted to the appropriate pH in distilled water. The media were sterilized by autoclaving before the bacteria were inoculated. The 0.01M buffer consisted of 0.9gl⁻¹ 3,3-dimethylglutaric acid puriss. (Koch-Light Laboratories Ltd, Colnbruck, Bucks); 1.95gl⁻¹ MES (2(N-morpholino)ethanesulfonic acid; 1.9gl⁻¹ ADA (N-(2-Acetamido)-Iminodiacetic acid; 2.38gl⁻¹ HEPES (N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; and 1.58gl⁻¹ TRIZMA HCl (Tris (hydroxymethyl aminomethane hydrochloride) (all Sigma Chemical Company, Poole). The pH was adjusted to 4.0, 4.5, 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 with 1M HCl (May & Baker Ltd, London) or 1M NaOH. The buffering capacity of the mixed buffer solution was found to be good over the range of pH required.

Duplicate tubes of the pH media, at each pH and for each species, were incubated at 15°C for 24 hrs. Growth was then estimated by measuring the absorbance of the cultures at 540 nm in a spectrophotometer (Unicam Instruments, Cambridge, England) and an average taken of the two tubes.

7.3.5 Growth, Polymer Production and Gliding Motility of the FLEXIBACTER sp. on Solid Media Containing a Selection of Carbon Sources and Nutrient Concentrations

The extent of gliding motility, and any relationship to polymer production, on media containing a range of nutrient concentrations and carbon sources was investigated.

(a) PYE medium agar was prepared as described in Section 2.3.2.

However, a range of peptone and yeast extract concentrations were used:

0.05% (w/v) peptone, 0.07% (w/v) yeast extract; 0.5% (w/v) peptone, 0.35% (w/v) yeast extract; 1% (w/v) peptone, 0.7% (w/v) yeast extract; and 2% (w/v) peptone, 1.4% (w/v) yeast extract.

(b) Minimal medium agar consisting of a range of carbon/nitrogen ratios and carbon sources were prepared as described previously (Section 4.3.2a and c). The media were glucose-limited, nitrogen-limited, glucose- and nitrogen-sufficient, or glucose/nitrogen sufficient with glycerol present or contained lactose, galactose, sucrose or mannose as the sole carbon source.

The Flexibacter sp. was streak inoculated onto duplicate plates of each medium. The cultures were then incubated at 15°C for 5 days before the extent of growth, polymer production, gliding motility and pigmentation was estimated. Gliding motility was observed using a binocular microscope (Olympus, Japan).

7.3.6 Bacterial Orientation at an Oil/Water Interface

Pure bacterial cultures were grown for 16 hrs at 15°C in PYE medium broth (Section 2.3.2) on a rotary incubator at 150 rpm. The cultures were then centrifuged at 10,960 *av.g* and the cells were resuspended to an optical density of 0.1 at 540 nm on a colorimeter (Corning Colorimeter 252) in 0.01M HEPES buffer (pH 7.4). Drops of cell suspension were then placed on a microscope slide and covered by a coverslip. 100 µl of hexadecane (Sigma Chemical Company, Poole) was slowly mixed with the cell suspension under the coverslip. The preparation was allowed to equilibrate for up to 15 min before observing cell orientation under a light microscope (Kyowa optical) at a magnification of x400.

7.3.7 Attachment assays

(a) Attachment Assays on Bacteria at Different Growth Phases

The four bacterial species were grown in PYE medium broth (Section

2.3.2) at 15°C on a rotary incubator at 150 rpm to exponential growth phase, stationary phase and death phase (72 hrs). (Appendix Table 5 for accurate times to early stationary phase).

Bacterial cultures were harvested by centrifugation at 10,960 av.g and washed once in 0.01M HEPES buffer, pH 7.4, before they were re-suspended in 0.01M HEPES, pH 7.4, to an optical density of 0.1 at 540 nm in a colorimeter.

The attachment assay procedure in Section 2.3.5 was used. Duplicate plates of PD and TCD for each isolate and growth phase were assayed after 60 min attachment.

The results were presented as $A_{590} (x10^{-3})$ of the attached cells on the surface and as an index of attachment (I_a). PD I_a and TCD I_a were calculated as described in Section 6.3.3(1).

(b) The Effect of Cell Density on Attachment

Pure cultures of the four bacterial species were grown in PYE medium broth (Section 7.3.2) for 16 hrs at 15°C on a rotary incubator. The cultures were harvested by centrifugation, washed once (Section 7.3.7) and resuspended in 0.01M HEPES (pH 7.4). The cells were resuspended to a range of different optical densities at 540 nm on a colorimeter. The densities were 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.3, 0.4 and 0.5 (except for E cloacae which was resuspended to a maximum optical density of 0.3).

Attachment was assayed (Section 2.3.5) using duplicate PD and TCD plates at each culture optical density. Duplicate PD and TCD plates of each optical density were incubated for 60 min before staining by the standard method. Results were presented as $A_{590} (x10^{-3})$ of attached cells.

(c) The Effect of pH on Attachment

Pure bacterial cultures were grown on PYE medium broth (Section

7.3.2). The bacteria were harvested and washed as described in Section 7.3.7. Cells were resuspended to an optical density of 0.1 at 540 nm in a colorimeter in the mixed buffer system (Section 7.3.4) which had been adjusted to pH of 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 or 9.5.

Standard attachment assay procedure (Section 2.3.5) was then followed by incubating at 15°C for 60 min. Results were expressed as $A_{590} (x10^{-3})$ of the crystal violet stained attached cells.

(d) The Effect of Temperature on Attachment

The four bacterial species were grown as described in Section 7.3.2 and harvested and washed using the procedures described in Section 7.3.7. Bacterial cells were resuspended in 0.01M HEPES buffer (pH 7.4) to an optical density of 0.1 at 540 nm in a colorimeter. The normal attachment assay procedure was then followed. However, duplicate plates of PD and TCD for each species were incubated for 60 min at a given temperature within a range of 4°C, 15°C, 20°C, 25°C, 30°C, 37°C and 45°C. The results were expressed as $A_{590} (x10^{-3})$ of attached cells.

7.3.8 Detachment Assays

The procedure for the detachment assays described in Section 5.3.4 was used. The 60 min detachment treatments were:

- (1) 0.01M HEPES buffer (pH 7.4)(control)
- (2) 5 µg/ml chloramphenicol (Sigma Chemical Company, Poole) in 0.01M HEPES buffer (pH 7.4)
- (3) 1% (w/v) sodium periodate (Fisons Scientific Reagents, Loughborough) in 0.01M HEPES buffer (pH 7.4)
- (4) 1 unit/5 ml protease (Sigma Chemical Company, Poole) in 0.01M HEPES buffer (pH 7.4)

The results were expressed as $A_{590} (x10^{-3})$ of attached cells and as PD I_d and TCD I_d , i.e. indices of detachment. I_d were calculated as the ratios of $A_{590} (x10^{-3})$ of the test surface to that of the appropriate

control surface. Any I_d greater or less than 1 represent an effect since values of 1 were recorded for treatments which had their 95% confidence limits of the mean overlapping with that of the control.

7.4 RESULTS

7.4.1 The Effect of Cell Density on the Attachment of the Four Bacterial Species

The relationship between bacterial cell concentration and levels of bacterial attachment were the same for all four bacterial species investigated (Figs. 7.1 - 7.4). Bacterial adsorption to the solid surfaces usually increased with increasing cell concentration until a maximum level of attachment was attained. After this maximum was reached there was little fluctuation and attachment remained more or less constant with further increases in cell concentration. Both the maximum levels of attachment and the maximum inoculum concentration achieving the plateau value of attachment varied with species and substratum. The attachment of Enterobacter cloacae did not reach a plateau, probably because the cell concentration of the inoculum did not reach a high enough level (Figs. 7.1 - 7.4).

7.4.2 The Effect of Growth Phase on Bacterial Attachment to Solid Surfaces

The effect of bacterial growth phase in batch culture on subsequent levels of bacterial attachment varied with the bacterial species and the attachment substratum. P. fluorescens (Table 7.1) showed no change in attachment to either surface with growth phase, and of the four bacterial species was the only one to show no change. The levels of adhesion of E. cloacae and the Chromobacterium sp, (Table 7.2 and 7.3) to PD was lowest in exponential phase cultures, and highest with stationary and death phase cells, with E. cloacae showing most attachment to PD in death phase. However, for these two species, with

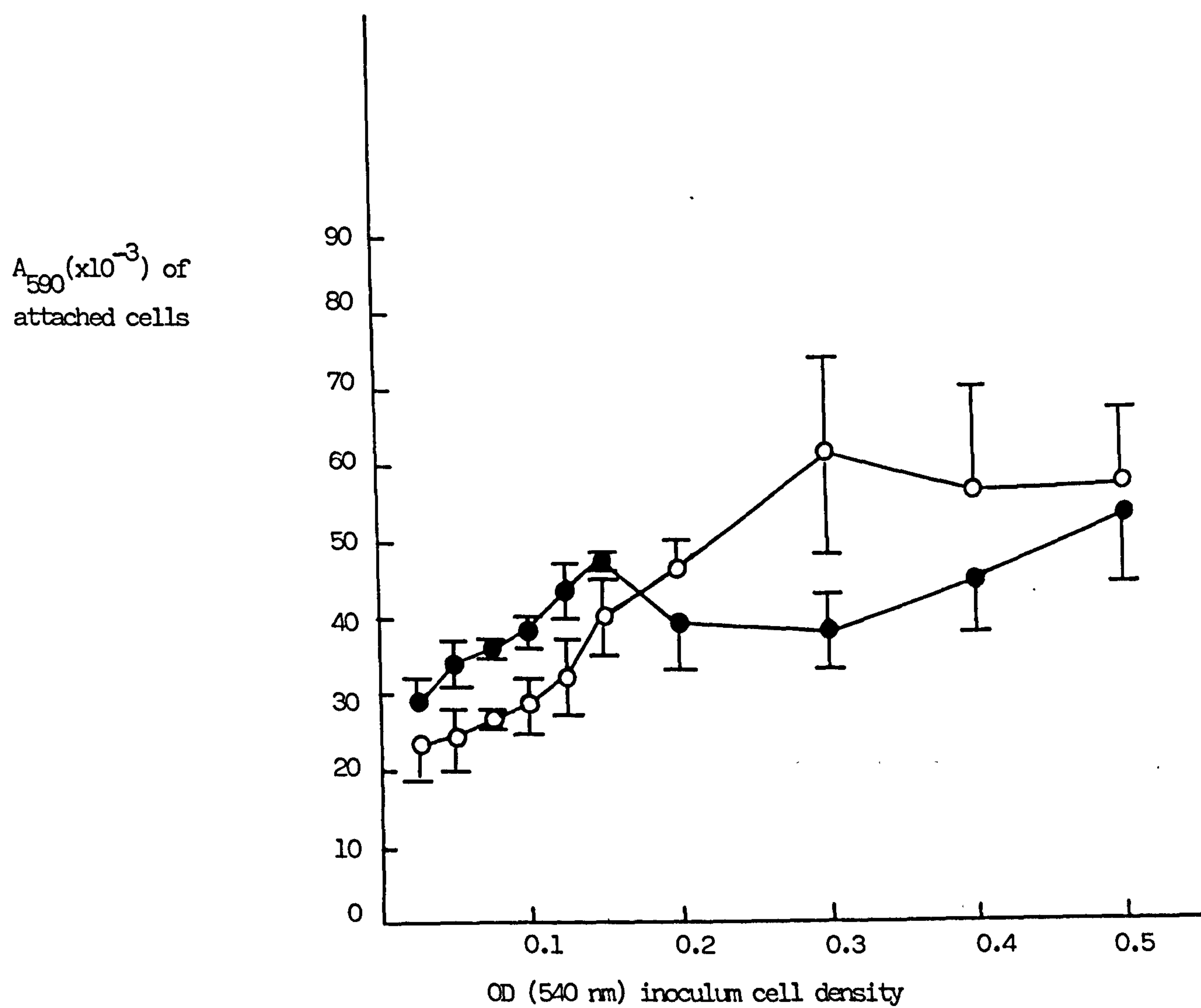


Figure 7.1 The effect of cell density on the attachment of Pseudomonas fluorescens to PD and TCD surfaces

(○), cells attached to PD surface; (●), cells attached to TCD surface

The bars represents the 95% confidence limits of the mean (n = 8)

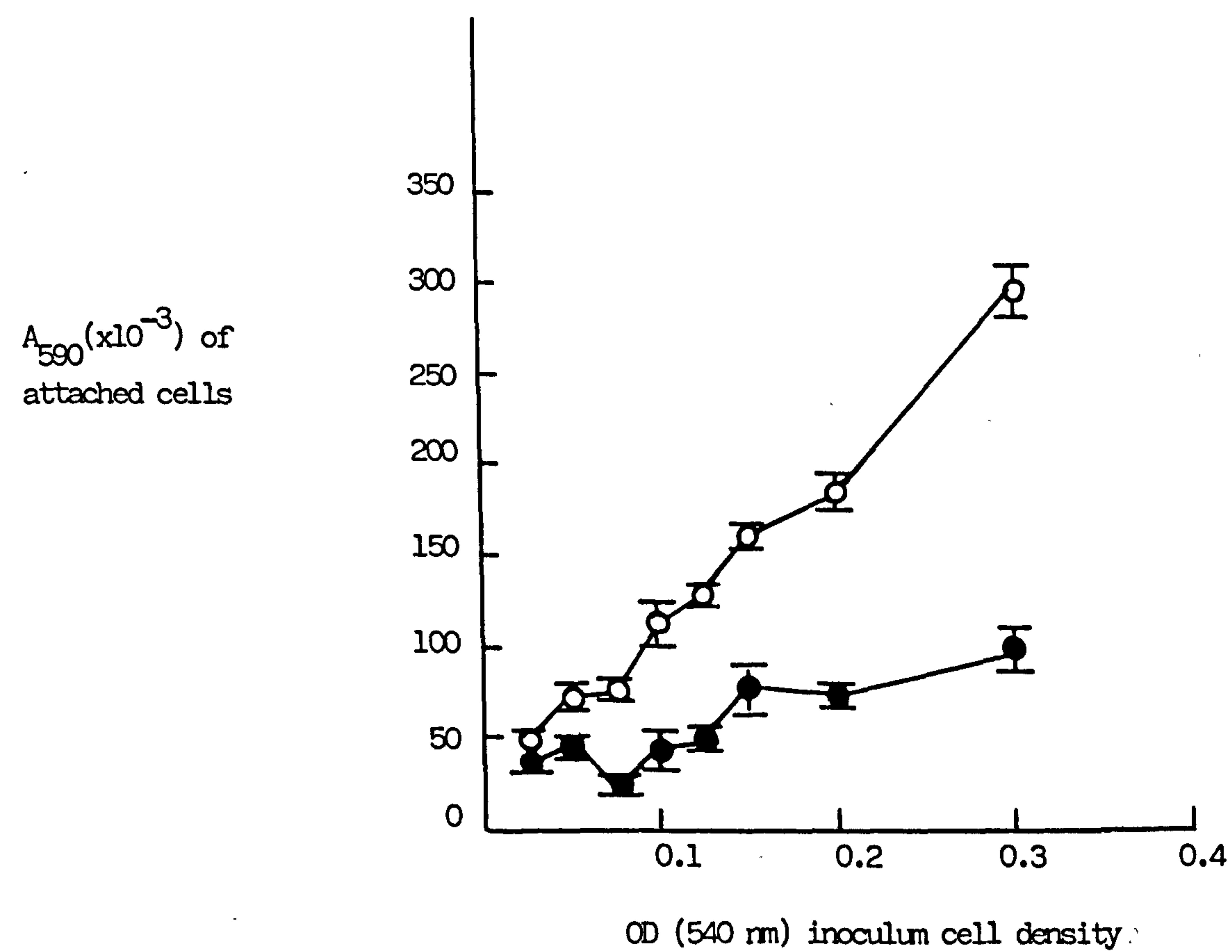


Figure 7.2 The effect of cell density on the attachment of Enterobacter cloacae to PD and TCD surfaces

(○), cells attached to PD surface; (●), cells attached to TCD surface
The bars represent the 95% confidence limits of the mean (n = 8)

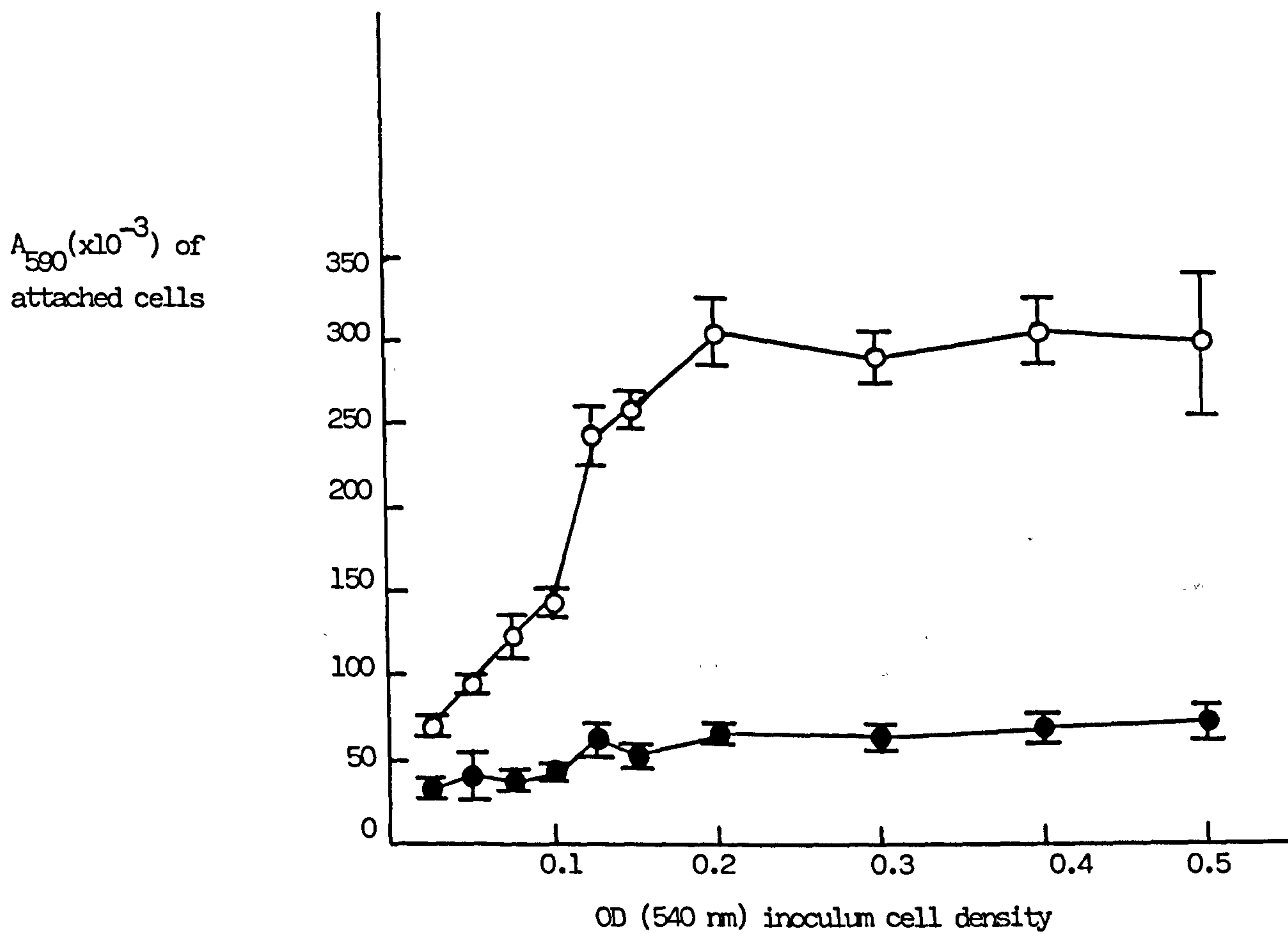


Figure 7.3 The effect of cell density on the attachment of the Chromobacterium sp to PD and TCD surfaces

(O), cells attached to PD surface; (●), cells attached to TCD surface

The bars represents the 95% confidence limits of the mean ($n = 8$)

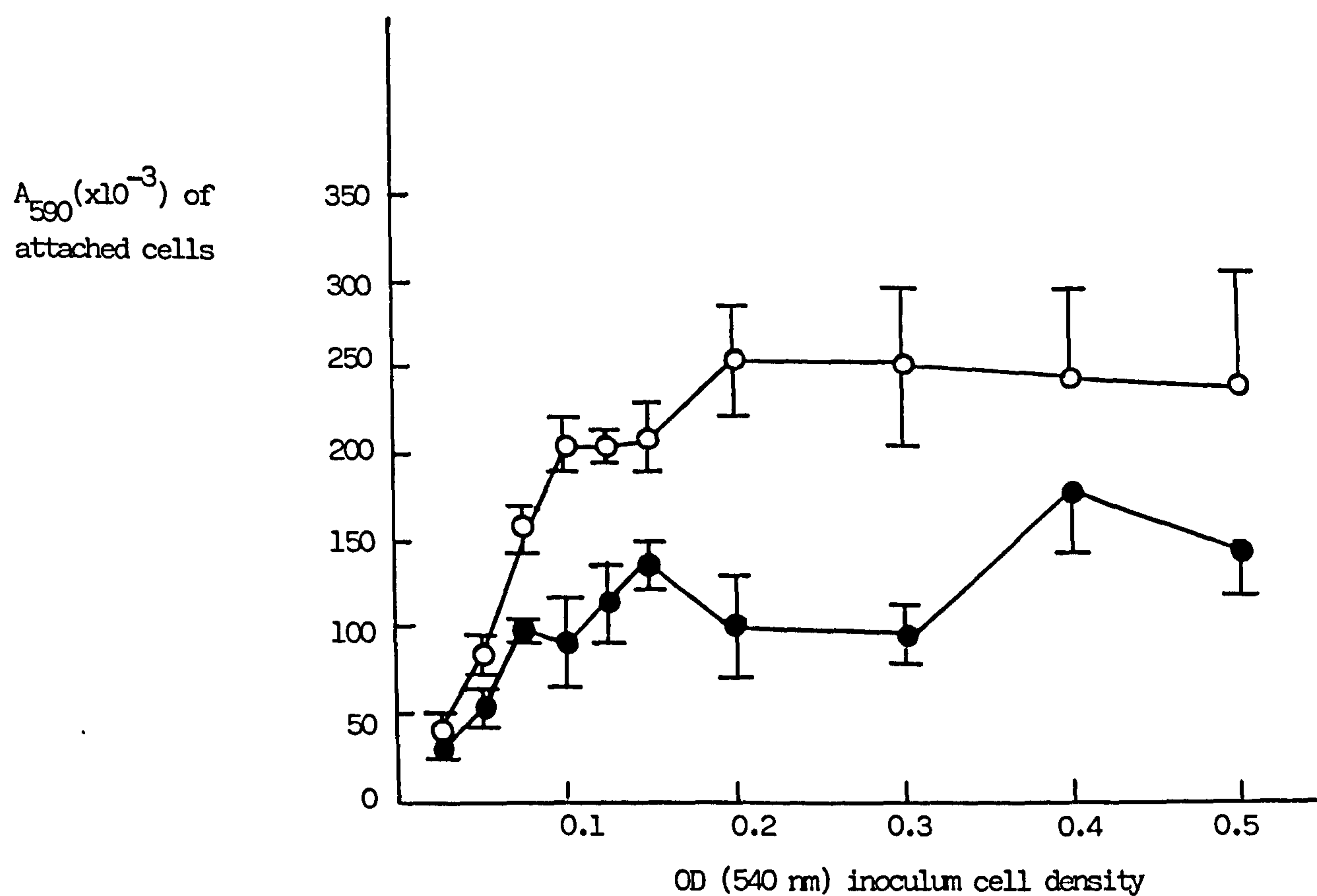


Figure 7.4 The effect of cell density on the attachment of the Flexibacter sp to PD and TCD surfaces

(\circ), cells attached to PD surface; (\bullet), cells attached to TCD surface

The bars represent the 95% confidence limits of the mean ($n = 8$)

TABLE 7.1 The Effect of Growth Phase in Batch Culture on the Subsequent Attachment of Pseudomonas fluorescens

Growth Phase	$A_{590}^{PD} (\times 10^{-3})$ attached cells	TCD	PD I_a	TCD I_a
Exponential	15 (± 2) ^a	27 (± 2)	1	1
Stationary	13 (± 0.8)	28 (± 4)	1	1
Death	15 (± 3)	27 (± 3)	1	1

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.2 The Effect of Growth Phase in Batch Culture on the Subsequent Attachment of Enterobacter cloacae

Growth Phase	$A_{590}^{PD} (\times 10^{-3})$ attached cells	TCD	PD I_a	TCD I_a
Exponential	19 (± 5) ^a	24 (± 3)	1	1
Stationary	42 (± 7)	27 (± 2)	2.2	1
Death	76 (± 10)	20 (± 3)	4	1

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.3 The Effect of Growth Phase in Batch Culture on the Subsequent Attachment of a Chromobacterium sp

Growth Phase	$A_{590}^{PD} (\times 10^{-3})$ attached cells	TCD	PD I_a	TCD I_a
Exponential	43 (± 3) ^a	13 (± 2)	1	1
Stationary	59 (± 3)	16 (± 4)	1.4	1
Death	57 (± 3)	20 (± 3)	1.3	1

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TCD there were no changes in attachment numbers with growth phase.

The attachment levels of the Flexibacter sp. (Table 7.4) to both surfaces, increased considerably after growth to stationary phase, but fell if the culture was allowed to reach death phase.

7.4.3 The Effect of Chloramphenicol, Sodium Periodate and Protease on Bacterial Detachment from Solid Surfaces

The inclusion of chloramphenicol (5 µg/ml), an inhibitor of protein synthesis, in detachment suspension did not cause loss of attached cells (Table 7.5 - 7.8). This was true for all four species and for both PD and TCD surfaces. Indeed, with P. fluorescens and E. cloacae the presence of chloramphenicol seemed to stabilize the film, reducing levels of detachment compared to the control (Tables 7.5 and 7.6).

In general, attachment to the TCD surface appeared to be more permanent and stable than that to the PD surface. Only P. fluorescens (Table 7.5) showed an increased detachment from TCD for only one treatment, that of protease (1 unit/5ml) - a proteolytic enzyme. Protease also removed cells of P. fluorescens, E. cloacae and the Chromobacterium sp. from the PD surface. Sodium periodate (1% w/v), which removes exopolysaccharide from the surfaces, detached both E. cloacae and the Chromobacterium sp. (Table 7.6 and 7.7) from the PD surface.

The detachment of the Flexibacter sp. (Table 7.8) did not increase with any of the treatments, remaining the same as the detachment control for both the PD and TCD surfaces.

7.4.4 The Orientation of Bacteria at an Oil/Water Interface

P. fluorescens, E. cloacae and the Chromobacterium sp. developed no permanent relationship or orientation at the hexadecane/water interface. Cells were not aligned in any specific manner, and tended to stream along the interface or exchange between the interface and the bulk water

TABLE 7.4 The Effect of Growth Phase in Batch Culture on the Subsequent Attachment of Flexibacter sp

Growth Phase	A_{590} (x10 ⁻³) attached cells		PD I_a	TCD I_a
	PD	TCD		
Exponential	157 (\pm 8)	44 (\pm 12)	1	1
Stationary	186 (\pm 26)	88 (\pm 9)	3.2	2
Death	25 (\pm 3) ^a	23 (\pm 0)	0.16	0.52

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.5 The Effect of Chloramphenicol, Sodium periodate and Protease on the Detachment of Pseudomonas fluorescens from PD and TCD Surfaces

Treatment	A_{590} (x10 ⁻³) attached cells		PD I_d	TCD I_d
	PD	TCD		
Control (1hr detachment)	10 (\pm 0.8) ^a	23 (\pm 3)	1	1
Chloramphenicol (5 ug/ml)	15 (\pm 3)	25 (\pm 2)	1.5	1.1
Sodium Periodate (1% w/v)	13 (\pm 3)	21 (\pm 5)	1	1
Protease (1 unit /5 ml)	4 (\pm 0.8)	14 (\pm 0.8)	0.4	0.6

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.6 The Effect of Chloramphenicol, Sodium periodate and Protease on the Detachment of Enterobacter cloacae from PD and TCD Surfaces

Treatment	A_{590} ($\times 10^{-3}$) attached cells		PD I_d	TCD I_d
	PD	TCD		
Control (1hr detachment)	17 (\pm 0.8) ^a	17 (\pm 2)	1	1
Chloramphenicol (5 ug/ml)	23 (\pm 2)	19 (\pm 0.8)	1.35	1
Sodium Periodate (1% w/v)	10 (\pm 2)	17 (\pm 0.8)	0.59	1
Protease (1 unit /5 ml)	5 (\pm 8)	20 (\pm 3)	0.29	1

^aParenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.7 The Effect of Chloramphenicol, Sodium periodate and Protease on the Detachment of Chromobacterium from PD and TCD Surfaces

Treatment	A_{590} ($\times 10^{-3}$) attached cells		PD I_d	TCD I_d
	PD	TCD		
Control (1hr detachment)	89 (\pm 5) ^a	14 (\pm 0.8)	1	1
Chloramphenicol (5 ug/ml)	83 (\pm 17)	13 (\pm 0.8)	1	1
Sodium Periodate (1% w/v)	68 (\pm 3)	17 (\pm 3)	0.76	1
Protease (1 unit /5 ml)	54 (\pm 5)	16 (\pm 0.8)	0.61	1

^aParenthetical values represent 95% confidence limits of the mean (n = 8)

TABLE 7.8 The Effect of Chloramphenicol, Sodium periodate and Protease
on the Detachment of a Flexibacter sp from PD and TCD
Surfaces

Treatment	A_{590} (x10 ⁻³) attached cells		PD I _d	TCD I _d
	PD	TCD		
Control (1hr detachment)	138 (\pm 6) ^a	45 (\pm 5)	1	1
Chloramphenicol (5 ug/ml)	132 (\pm 17)	53 (\pm 3)	1	1
Sodium Periodate (1% w/v)	142 (\pm 9)	48 (\pm 7)	1	1
Protease (1 unit /5 ml)	127 (\pm 11)	41 (\pm 5)	1	1

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

phase (Fig. 7.5(a)).

The Flexibacter sp. presented a different picture. The cells often became orientated at 90° to the interface in an 'end on' arrangement. Once associated with the hexadecane phase the Flexibacter sp. did not desorb from the interface and there was little exchange from the interface to the bulk phase (Fig. 7.5(b)). On occasions, if small droplets of hexadecane were mixed in the bulk aqueous phase, it was possible to see individual Flexibacter cells with small globules of hexadecane at each pole.

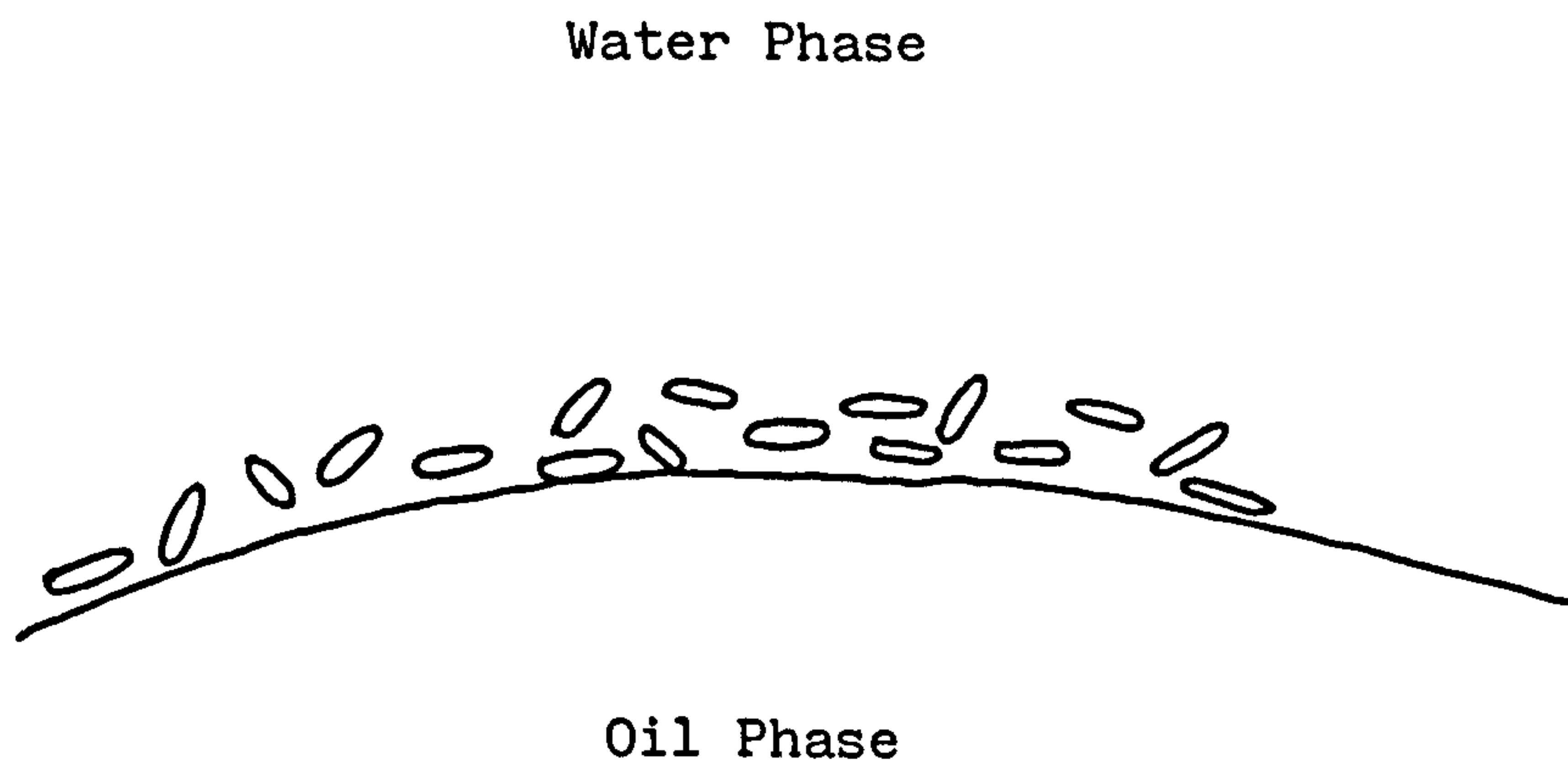
7.4.5 The Effect of pH on the Attachment of Bacteria to Surfaces

The attachment of the three non-gliding bacteria showed similar changes with change in pH. There were peaks in attachment at a pH within pH 5.5 and 7.0 for P. fluorescens, E. cloacae and the Chromobacterium sp; the size of these attachment peaks varied with bacterial species and the nature of the substratum. There were consistent reductions in attachment with extreme pH's, i.e. below pH 5 and above pH 8 (Fig. 7.6). The levels of growth for P. fluorescens, E. cloacae and the Chromobacterium sp, all were maximum between pH 5 and 8.5 (Fig. 7.7). Peaks in attachment did not necessarily correspond to peaks in growth levels at a given pH, though there was some correspondence for P. fluorescens, E. cloacae. Overall bacterial physiology was probably not a direct influence on levels of attachment (Fig. 7.7).

The gliding bacterium, Flexibacter sp, showed an entirely different attachment profile in the range of pH solutions to that of any of the other bacteria. The attachment of this bacterial species to both PD and TCD surfaces steadily declined with increasing pH (Fig. 7.6). There was no relationship between pH effects on attachment and on growth, since the Flexibacter sp, as the other three bacteria, showed maximum growth at neutral pH's (Fig. 7.7).

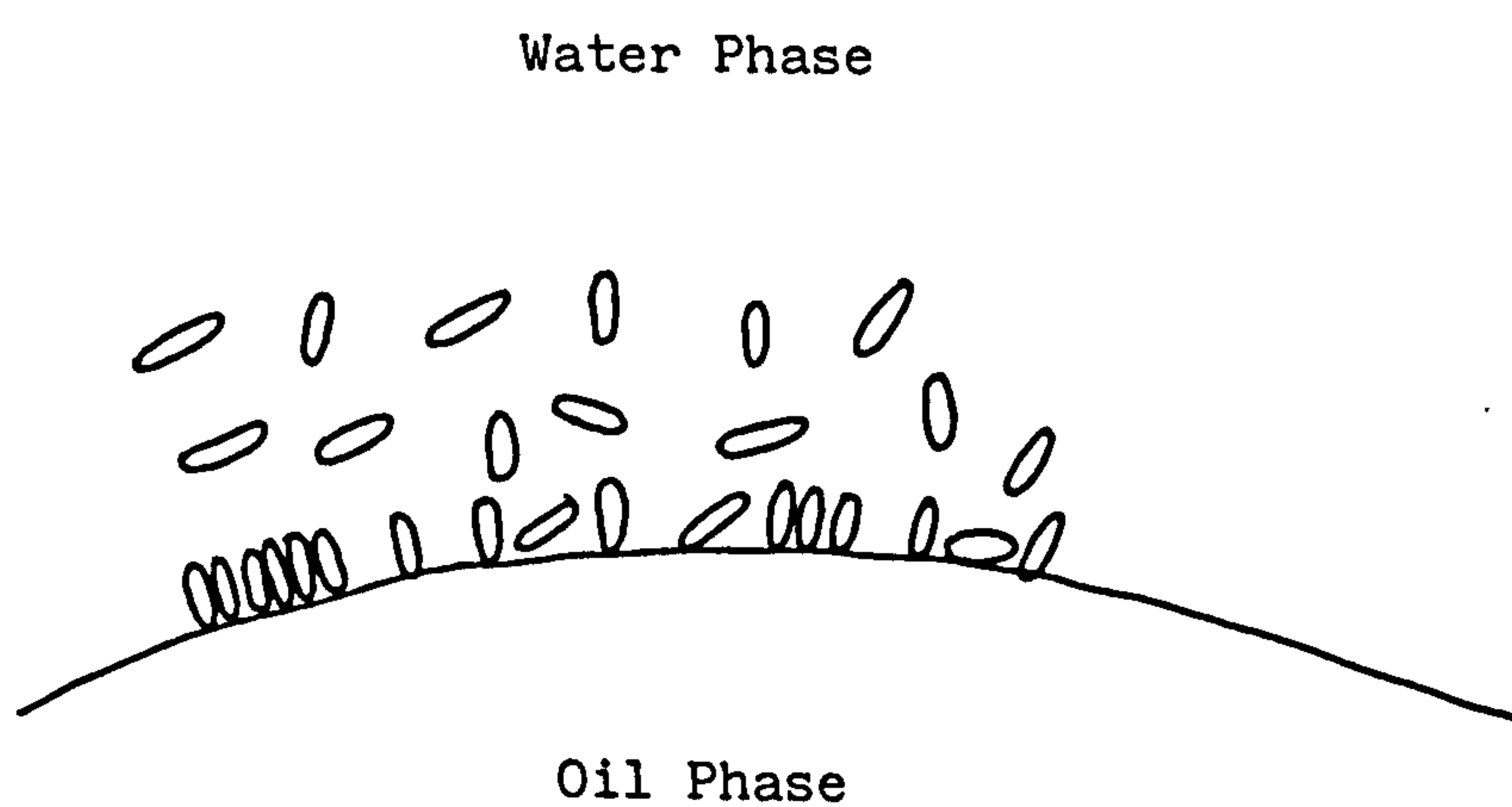
Figure 7.5 The orientation of the four selected species of bacteria at an oil/water interface

(a) Pseudomonas fluorescens, Enterobacter cloacae and Chromobacterium sp



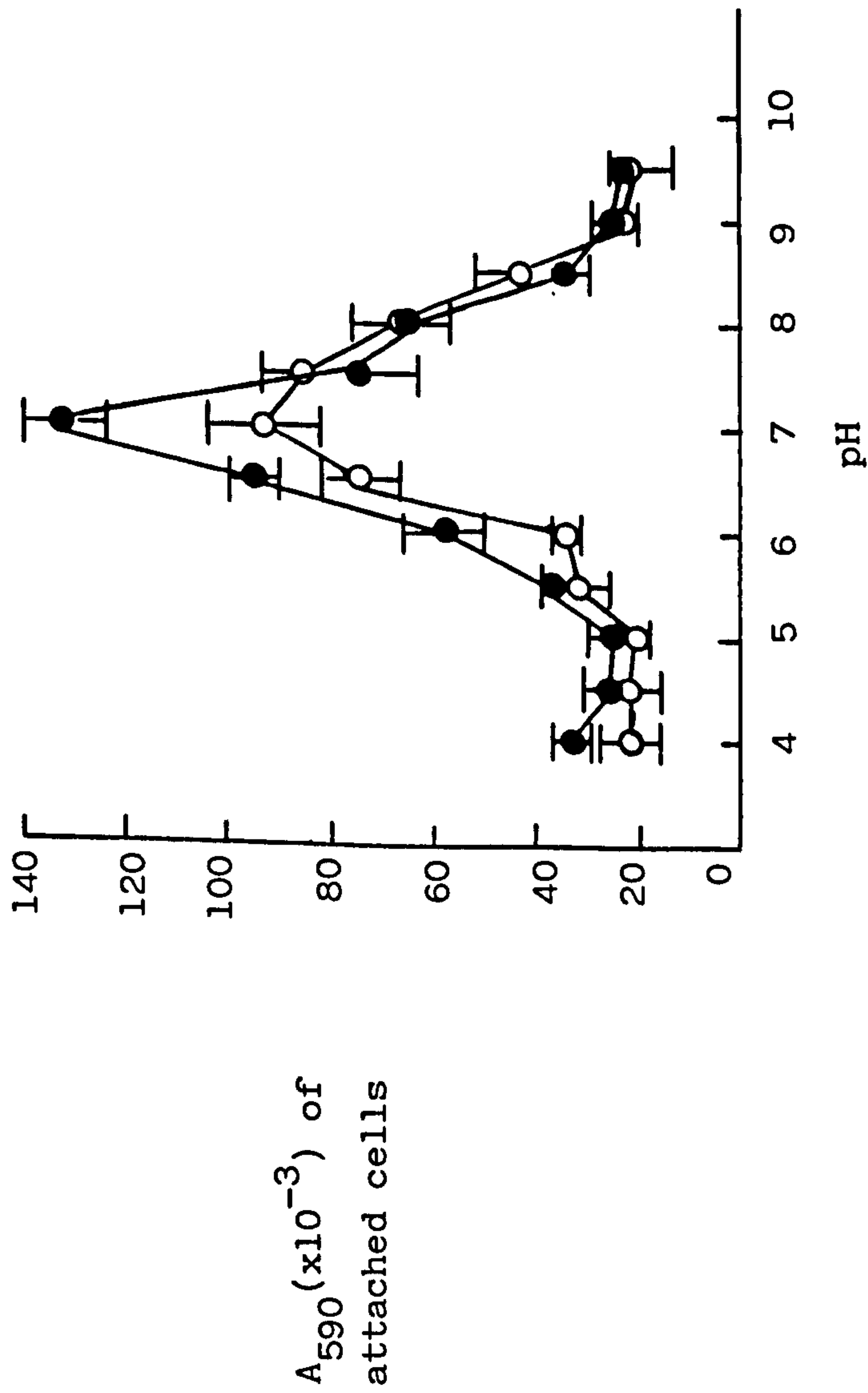
No permanent orientation at or association with the interface

(b) Flexibacter sp

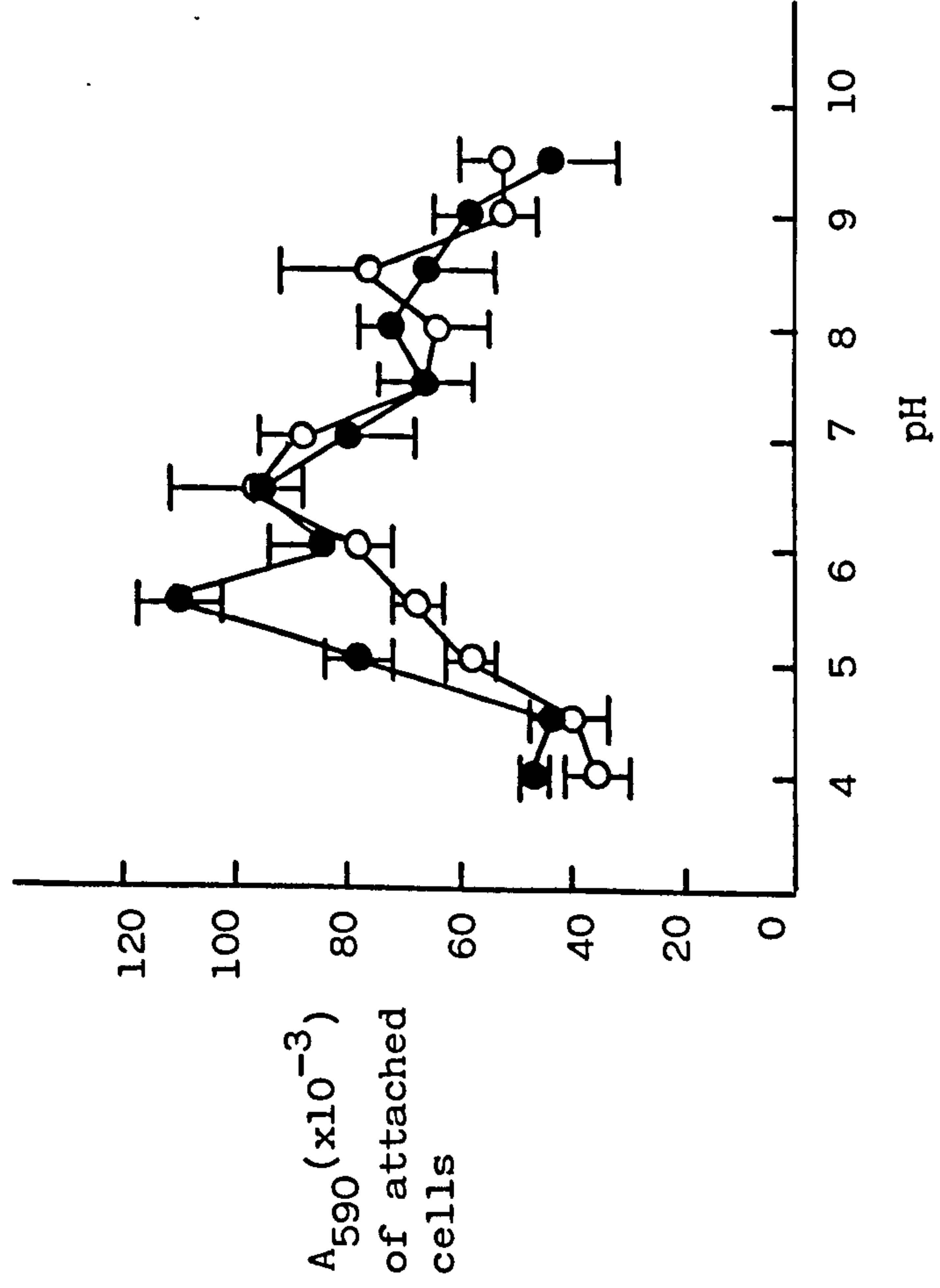


End on orientation towards the oil phase, fairly stable

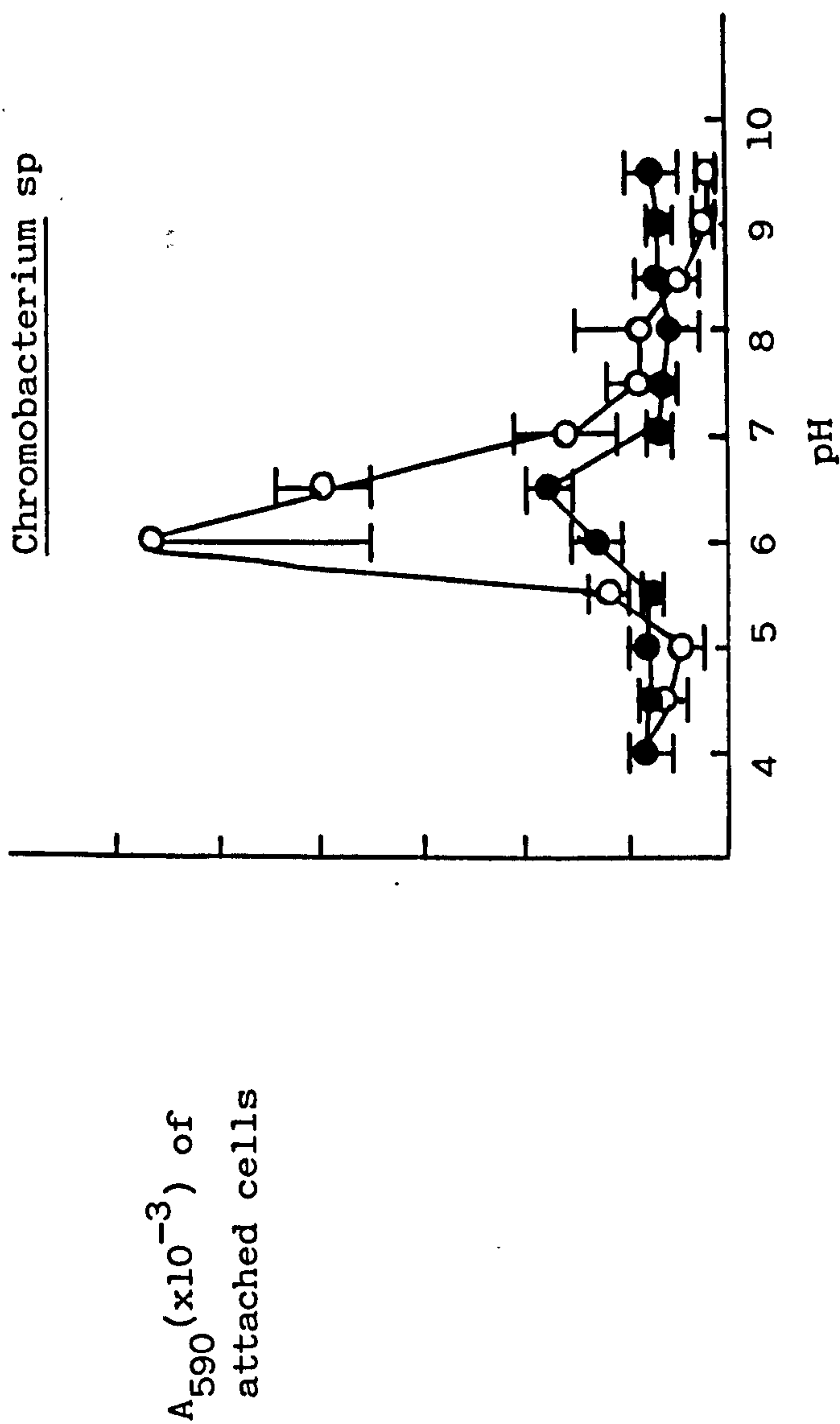
Pseudomonas fluorescens



Enterobacter cloacae



Chromobacterium sp



Flexibacter sp

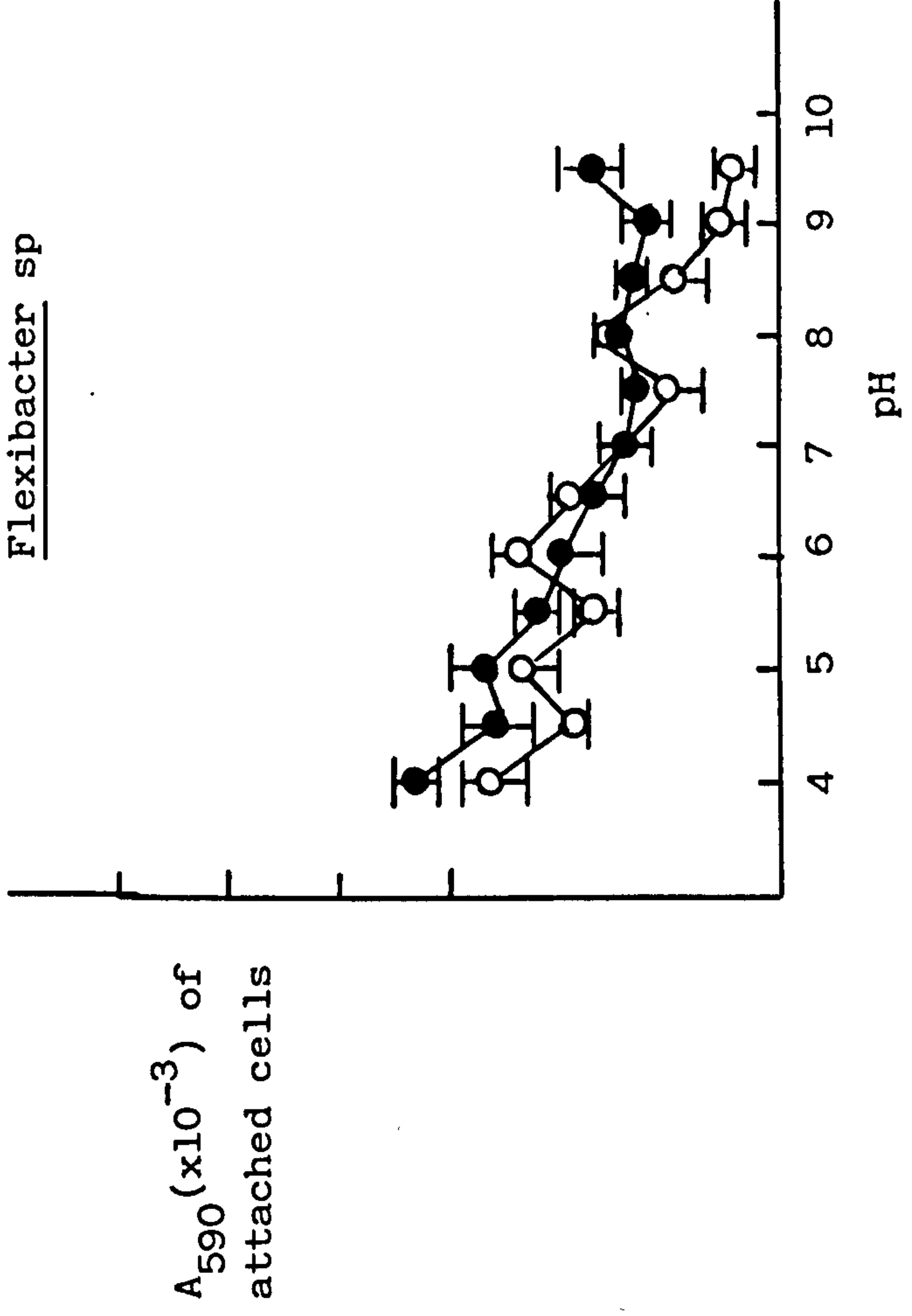


Figure 7.6 The effect of pH on bacterial attachment to PD and TCD surfaces (O), cells attached to PD surface; (●), cells attached to TCD surface. The bars represent the 95% confidence limits of the mean (n = 8)

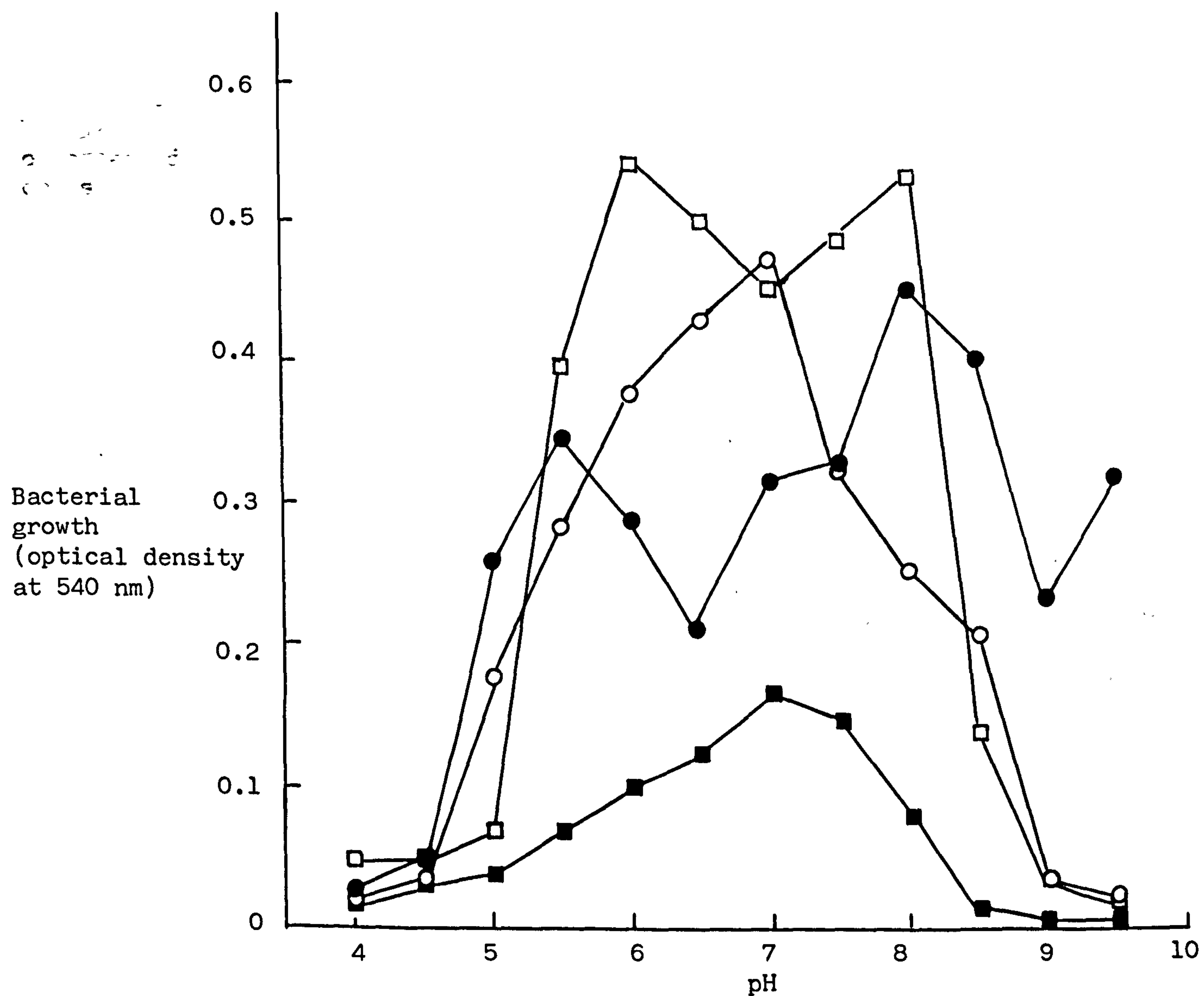


Figure 7.7 The effect of pH on bacterial growth

(○), *Pseudomonas fluorescens*; (●), *Enterobacter cloacae*;
 (□), *Chromobacterium* sp.; (■), *Flexibacter* sp.

7.4.6 The Effect of Temperature on the Attachment of Bacteria to Surfaces

Again there was a considerable difference in the way the three non-gliding bacteria and the gliding bacterium responded in their attachment levels with changes in incubation temperature (Fig. 7.8). The P. fluorescens, E. cloacae and the Chromobacterium sp, all showed peaks in attachment to both surfaces within a temperature range of 20-25°C. The maximum levels of attachment varied with bacterial species and substratum and the degree of the decline in attachment at the extreme temperatures also varied with the bacterial species (Fig. 7.8). The Flexibacter sp, however, showed no peak in attachment but rather a steady decline with increasing temperature, and was minimal for both PD and TCD surfaces at 45°C.

The range of temperatures over which the four bacterial species showed good growth (Table 7.9) was so broad that it is impossible to conclude a direct relationship between growth/activity and attachment.

7.4.7 The Effect of Nutrient Concentration and Nutrient Source on the Gliding Motility and Polymer Production of the FLEXIBACTER sp on Solid Media

The extent of polymer production and gliding motility by the Flexibacter sp, was directly related to nutrient concentration and the nutrient source. As the nutrient concentration of PYE medium agar (Table 7.10) increased above 0.5% (w/v) peptone and 0.35% (w/v) yeast extract, so gliding motility by the Flexibacter sp, across the agar surface ceased. This was associated with the increased production of a mucoid viscous polymer. The colonial form developed at the lower nutrient concentrations was spreading, and the bacteria demonstrated gliding motility at the colony edges and the colonies were not associated with the mucoid polymer.

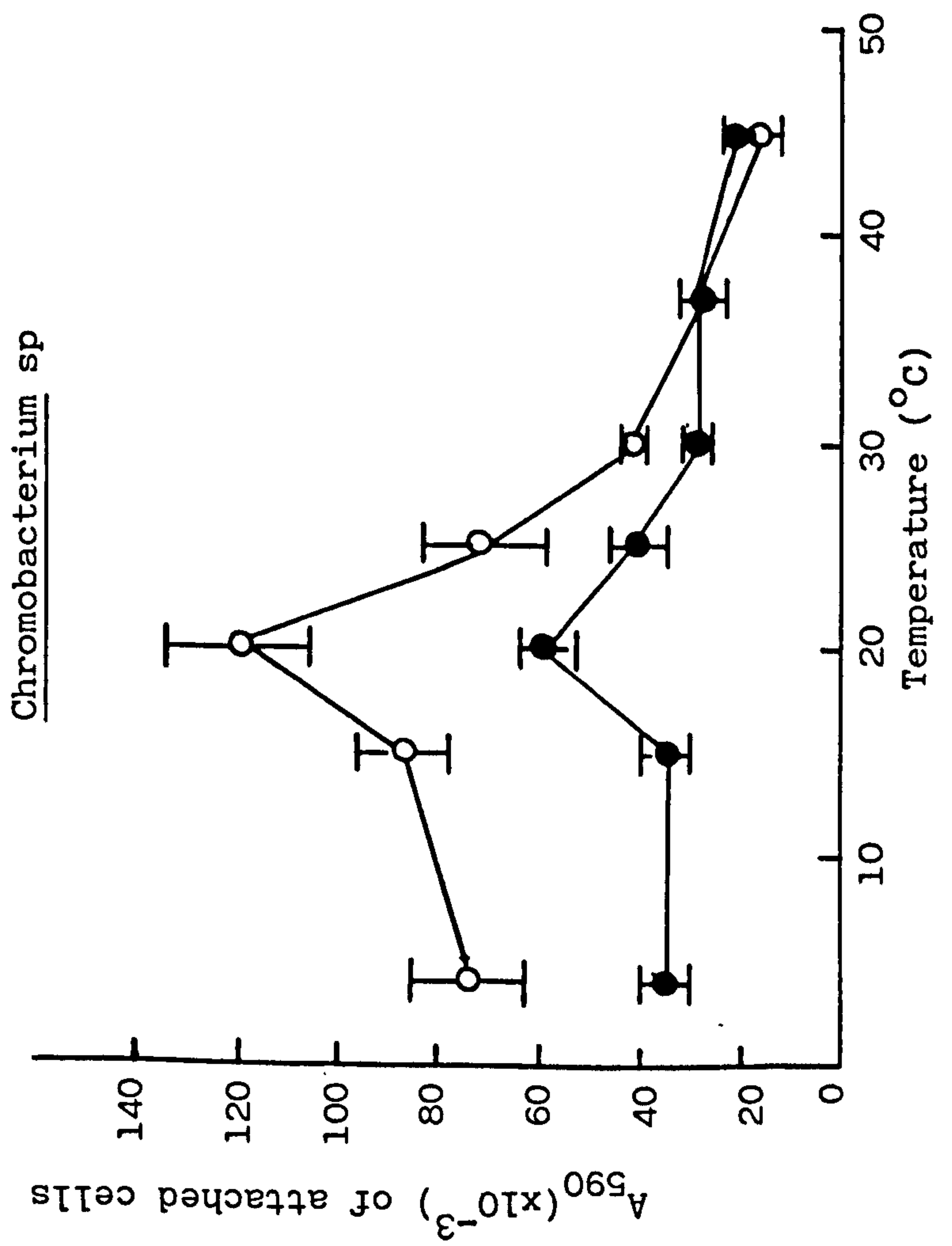
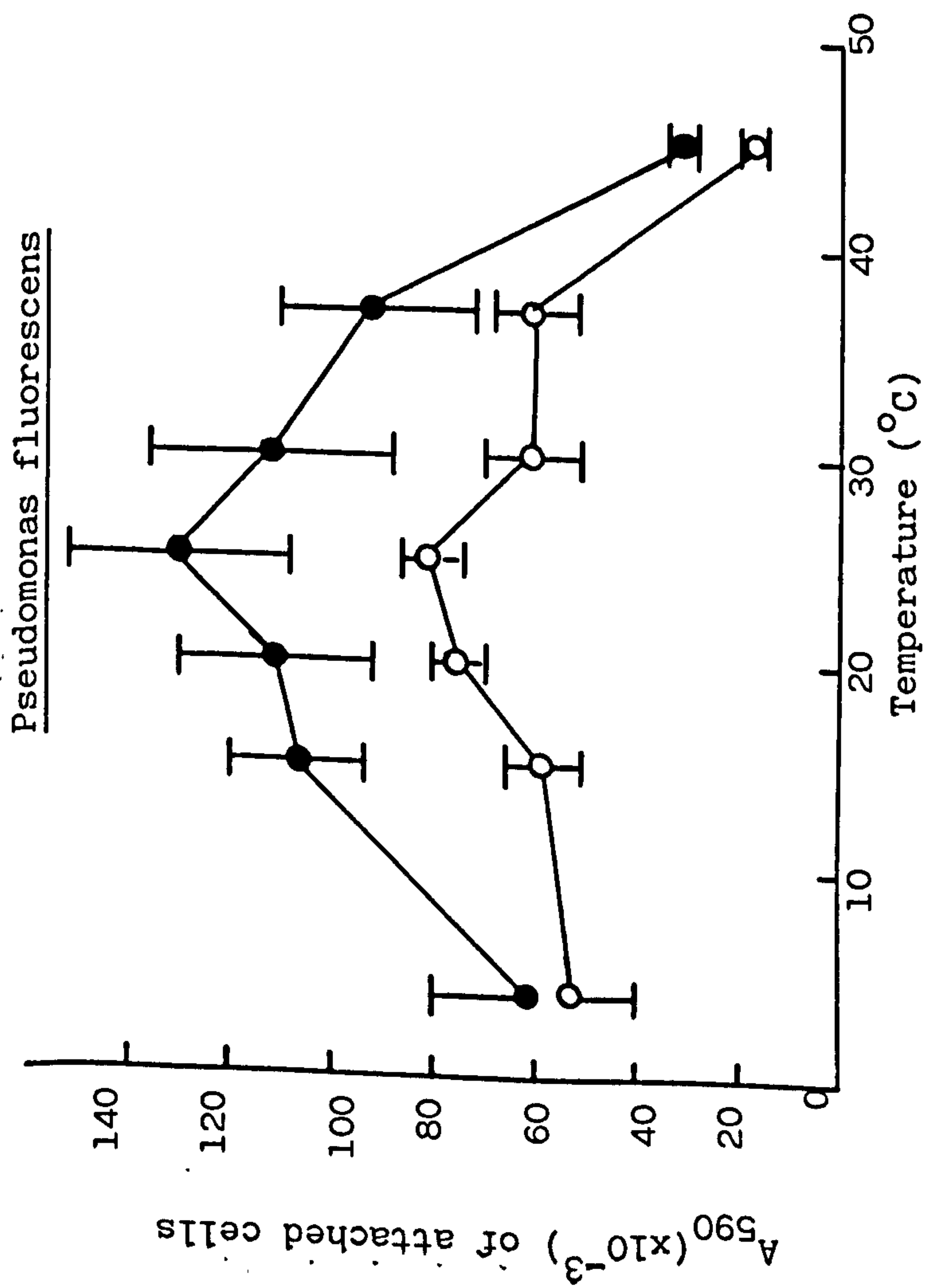
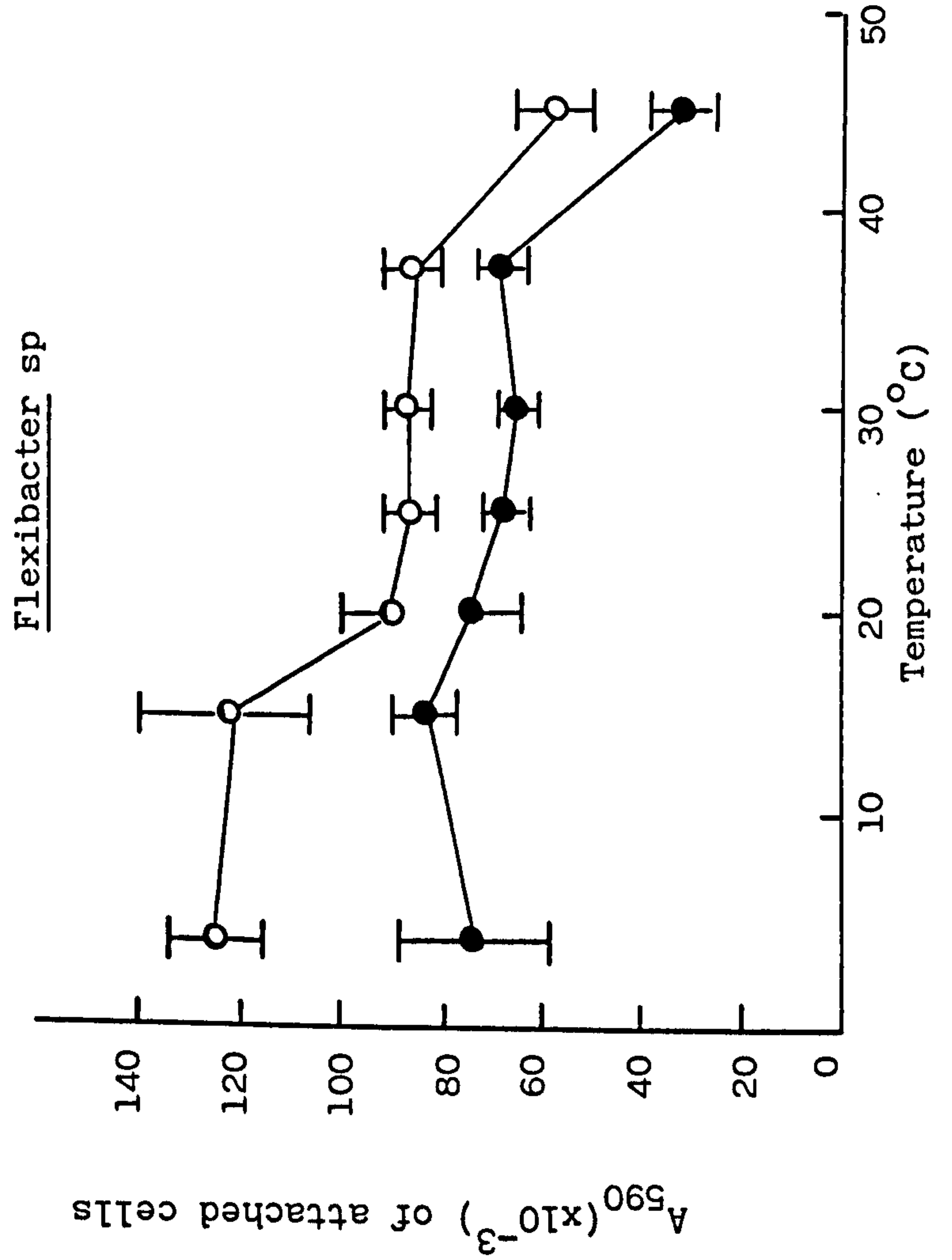
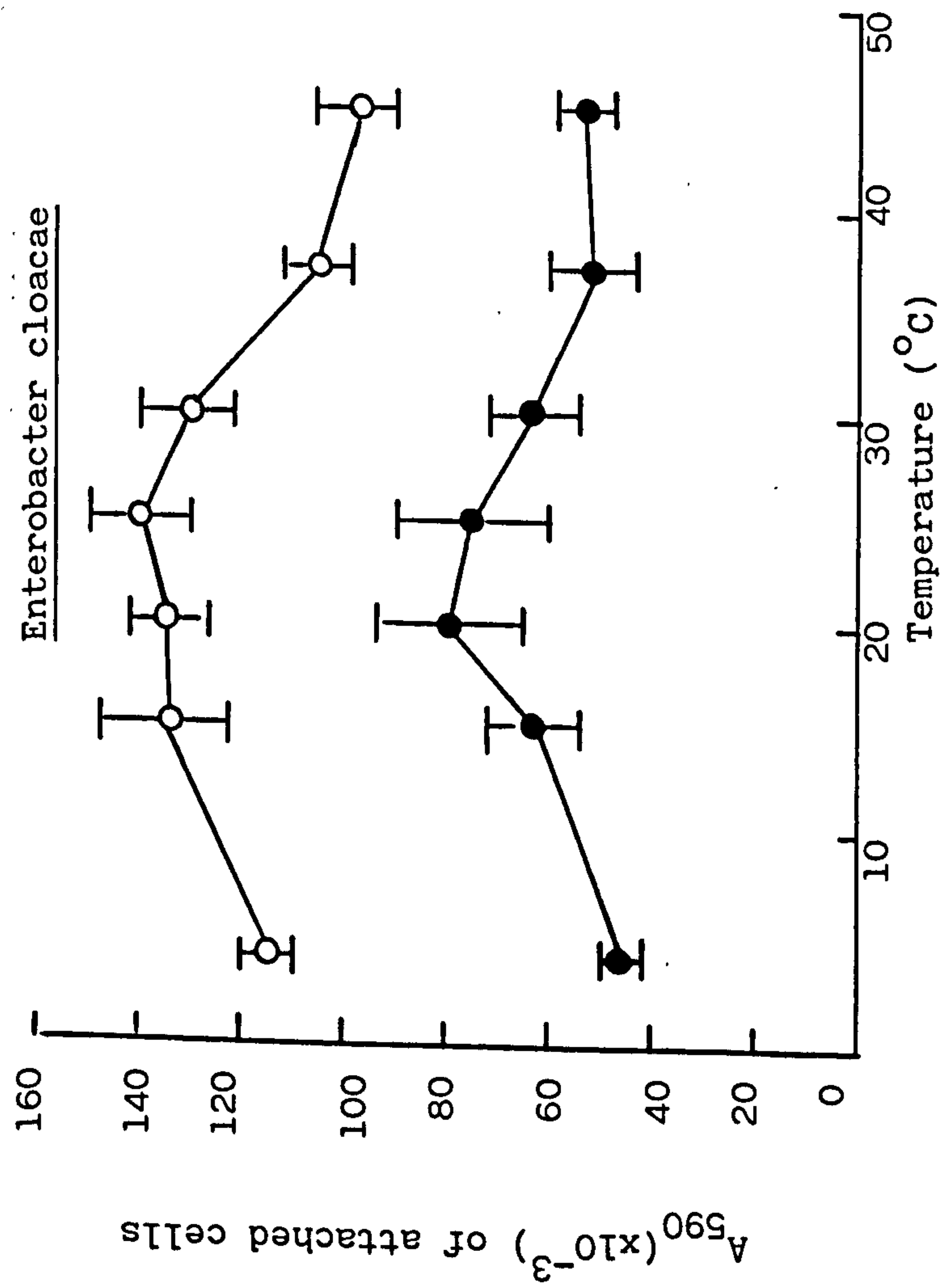


Figure 7.8 The effect of temperature on bacterial attachment to PD and TCD surfaces. (O), cells attached to PD surface; (●), cells attached to TCD surface. The bars represent the 95% confidence limits of the mean (n = 8)

TABLE 7.9 The Effect of Temperature on the Growth and Motility of
4 Bacterial Species

Temperature(°C)	Pseudomonas fluorescens	Enterobacter cloacae	Chromobacterium sp	Flexibacter sp
	(a) G ⁺ _(e)	(b) M ⁺ ₋		
4	G ⁺	M ⁺ ₋	G ⁺	M ⁺
15	G ⁺⁺	M ⁺	G ⁺⁺	M ⁺⁺
20	G ⁺⁺	M ⁺	G ⁺⁺	M ⁺⁺
25	G ⁺⁺⁺	M ⁺	G ⁺⁺	M ⁺⁺
30	G ⁺⁺	M ⁺⁺	G ⁺	M ⁺⁺
37	NG(c)	G ⁺⁺	G ⁺	M ⁺ ₋
45	NG	G ⁺ ₋	G ⁺ ₋	M ⁺ ₋

^aG = growth, ^bM = motility, ^cNG = no growth, ^dNM = no motility

^e₋ = poor, + = fair, ++ = moderate, +++ = good growth or motility

TABLE 7.10 The Effect of Nutrient Concentration and Nutrient Source on the Gliding Motility and Polymer Production of the Flexibacter sp on Agar Media

Nutrient Source and Nutrient Conc.	Growth	Colony Type	Gliding Motility	Polymer Product ⁿ	Pigment Product ⁿ
0.05% (w/v) P(a) 0.035% (w/v) YE(b)	+++ ^(f)	SP ^(c)	++	0	±
0.1% (w/v) P 0.07% (w/v) YE	+++	SP	+++	±	+
0.5% (w/v) P 0.35% (w/v) YE	+++	(d) SP L/edge colony	V ⁺	++	++
1% (w/v) P 0.7% (w/v) YE	+++	L	0	+++	+++
2% (w/v) P 1.4% (w/v) YE	+++	L	0	++++	++++
Glucose limited	+++	(e) S	0	+	+
Nitrogen Limited	+++	L	0	+++	++
Glucose & Nitrogen Sufficient	+++	L	0	+++	+++
Glucose & Nitrogen & Glycerol Sufficient	+++	L	0	+++	+++
Lactose	+++	L S/SP	L 0 S +	L +++ S 0	L +++ S ±
Galactose	+++	L	0	+++	+++
Sucrose	+++	S	0	±	±
Mannose	+++	L	0	+++	+++

^aP = peptone; ^bYE = yeast extract; ^cSP = spreading colonies;
^dL = colonies > 1mm diameter; ^eS = colonies < 1 mm; ^f± = poor;
 + = fair; ++ = moderate; +++ = good; ++++ = very good gliding growth, polymer production or pigment production

The different carbon/nitrogen ratio and carbon source media did not induce motility, but some of the media promoted the production of large amounts of polymer. However, two carbon source media differed in their effects from this generalization. Firstly, sucrose caused the production of little polymer but showed no concomitant increase in the ability of the cells to undergo a gliding motility across the agar surface. Secondly, lactose as the sole carbon source caused the development of two colonial types, the first producing large amounts of the mucoid polymer and showing no gliding motility; the second producing no mucoid polymer and demonstrating weak gliding motility (Table 7.10). On subculture to fresh lactose medium, each of the colonial variants again gave rise to the two forms. However, the small colonies producing little polymer appeared to be less viable than the mucoid form since fewer colonies developed from similar sized inocula of the small colonial form compared to the mucoid form.

(Appendix Tables 23 to 26 show details of repeat experiments for the effect of growth phase, detachment treatments, temperature and pH on levels of bacterial attachment and detachment).

7.5 DISCUSSION

7.5.1 The Role of Bacterial Cell Concentration on Bacterial Attachment

The attachment of the three non-gliding bacteria and the Flexibacter sp, showed similar relationships between suspended cell numbers and attached numbers. Attachment increased with cell concentration, reaching a plateau value for 3 species. For each solid surface and bacterial species there were variations in the rates of attachment and the final plateau level. Similar results have been described for the effect of time on the attachment (Chapter 4) of the four species investigated. The explanation for these effects probably resides in the

fact that increased attachment time and cell concentration will lead to an increased probability of contact, i.e. collisions between the bacteria and the substratum surfaces. This will increase the opportunities for bacterial attachment interactions with the solid surface. The different attachment plateau levels demonstrated by the bacteria for the surfaces may, however, reflect differences in adsorption capacities of the substrata for the individual species.

Since all the bacteria investigated showed similar changes in attachment levels with increases in cell concentration or time, culminating in a plateau level, similar types of physico-chemical interactions may well be involved in the permanent attachment of all four species.

7.5.2 The Importance of Bacterial Growth Phase and Microbial Activity on Attachment

Changes of bacterial growth phase in batch culture have often been shown to be reflected in differences in bacterial attachment to solid surfaces (Zvyagintsev, 1973; Fletcher, 1977; Minato & Suto, 1979). The actual effects have been found to vary considerably with species. These differences for the majority of bacteria may be linked to changes in bacterial cell surface characteristics rather than to a direct effect, such as metabolic activity (Chapter 4). The results presented in this Chapter for the attachment of P. fluorescens, E. cloacae and the Chromobacterium sp, demonstrate the variability in levels of attachment with growth phase. Each bacterium interacts differently, and the solid substratum further modifies the attachment. In all three non-gliding bacteria attachment to the TCD surface was unaltered by the changes in growth phase while those to the PD surface could be considerably affected. This underlines the probable importance of physico-chemical interactions for these species. The effect is probably caused by

bacterial cell surface properties involved in adhesion changing with the bacterial growth phase. The solid surface, however, acts as a further mediator in the physico-chemical interaction dictating the types of interactions possible, e.g. the PD surface would provide more opportunity for hydrophobic bonding than the TCD surface.

None of the three non-gliding bacteria showed any decline in attachment in death phase (Tables 7.1-7.3), and indeed E. cloacae showed a rise in levels of attachment to the PD surface in this growth phase. Other workers have found that some bacteria undergoing permanent adhesion decrease their attachment to surfaces in death phase (Minato & Suto, 1979; Fletcher, 1977). However, the declines were normally neither rapid nor major. The results for the Flexibacter sp, presented above showed two differences from the other species. First, there was a consistent rise in attachment in stationary phase for both TCD and PD surfaces; second, there was a rapid and large decline in attachment to both surfaces when the cells were in death phase (Table 7.4).

The Flexibacter sp, then reacts very differently to a substratum than the other three bacteria. Its major decline in attachment with death phase suggests the possibility that bacterial activity may be a requirement for the attachment of Flexibacter sp. This was supported by results presented in Chapter 4 which showed a rise in the attachment of Flexibacter sp, with increasing growth rate, a feature not demonstrated by any of the other bacteria. The role played by physiological activity in the adhesion of this bacterium may be due to an increased accumulation of a surface constituent, e.g. polymer supporting attachment or due to an active response, e.g. the production of an adhesive polymer, once contact with a substratum has occurred. Attachment may be high in stationary phase cells either because the production of, or the accumulation of, such a constituent may be

maximum. Death phase cells may have lost their ability to produce and/or accumulate the substance.

7.5.3 The Role of the Hydrophobic Poles in the Attachment of the FLEXIBACTER sp

The gliding bacterium was demonstrated as orientating perpendicularly at an oil/water interface, this probably relates to the polar region of its cells being hydrophobic in nature (Chapter 5).

Flexibacter aurantiacus CW7 and Hyphomicrobium vulgare ZV580 were shown to align in a similar manner at an air/water, an oil/water and a solid/water interface by Marshall and Cruickshank (1973).

Interestingly, the Flexibacter sp, investigated in this Chapter may have been hydrophobic at both poles since hexadecane droplets accumulated on occasions, at both ends of the cells. None of the other bacteria showed such interactions in this two phase system, i.e. hexadecane/water. The hydrophobicity of the poles of the Flexibacter sp, may impart an advantage in the initial association with a surface, maintained and supplemented by the effects of microbial activity. These attachment interactions are probably still within the framework of physico-chemical phenomena, involving both hydrophobic and charge interactions (Chapter 5).

Marshall and Cruickshank (1973) found that Flexibacter aurantiacus CW7 when orientated perpendicularly at a solid/water interface, had a polymeric material 'bridging' a gap between the substratum and the bacterium. They were unsure whether the bacterial cell surface or the polymeric material contributed to the hydrophobic nature of the cell poles. However, several gliding bacteria including one Flexibacter sp, have been shown to possess an unusual group of sulfonolipids in their cell envelopes (Godchaux III & Leadbetter, 1983) and it may be these that contribute to pole hydrophobicity. However, hydrophobic bonding

does not dominate the attachment interactions of the Flexibacter sp (Chapter 5) and attachment of the species increased with activity even though there was a decline in cell surface hydrophobicity. It may be that the production of a bridging polymer is the major determinant of attachment. The hydrophobic poles of the cells may draw the Flexibacter sp, to the surface where further association is cemented by the production of polymer, with the cell eventually coming to lie flat on the surface and undergoing permanent attachment to the substratum.

7.5.4 The Detachment of Three Non-Gliding and a Gliding Bacterium from Substrata

Major differences were again demonstrated between the gliding and the non-gliding bacteria in their detachment profiles. The Flexibacter sp, was generally less likely to detach. Once attached to a solid substratum, protein synthesis did not appear to be a significant feature in maintaining the adhesion for any of the bacteria investigated because chloramphenicol had no effect. The inclusion of inhibitors of protein synthesis such as chloramphenicol has been shown to inhibit the irreversible sorption of marine pseudomonads, however, Hyphomicrobium attachment was unaffected by either chloramphenicol or streptomycin (Marshall, 1974). It therefore, appears that not only does the effect vary with bacterial species, but it may also with the stage of attachment that the inhibitors are applied. Pre-incubation with protein inhibitors may change bacterial surface characteristics and consequently their attachment. The stability of the attachment after post-attachment incubation with inhibitors indicates that the attachment interactions may be permanent in that they do not necessarily require further stabilization by the production of fresh material or the maintenance of activity by cells.

The effect of sodium periodate, which denatures polysaccharides by

oxidizing and cleaving adjacent vicinal hydroxyl groups, characteristic of carbohydrate molecules and protease, differ between the three non-gliding bacteria and the Flexibacter sp, and with solid substratum. Apparently both protein and exopolysaccharides were involved in the attachment of E. cloacae and the Chromobacterium sp, since both protease and sodium periodate increased rates of bacterial detachment. This was demonstrated on only one of the solid substrata, i.e. PD, and indicates that the interactions between PD and TCD solid surfaces and the bacterial cells may be different. A similar conclusion was drawn by Fletcher and Marshall (1982). The data presented in this Chapter indicates that attachment to the TCD substratum appears to be firmer than that to the PD surface, possibly because of the increased bonding capacity of TCD over PD (Table 5.9). These differences may be due to actual variations in the nature and type of bonds between the substratum and the bacterial surface perhaps with different constituents on the bacterial cell surface involved, or they may be due to the association with the TCD surface being far closer and the configuration less accessible to disruption by external agents such as enzymes. P. fluorescens showed detachment from both surfaces in the presence of protease but not with periodate. This may suggest that proteins and not exopolysaccharides are significant contributors to the attachment of this species. However, it should be noted that periodate does not degrade all carbohydrates, and that again there might be some configurational protection. The adhesion of the Flexibacter sp, to both surfaces was unaffected by either protease or periodate. This may indicate either that proteins and exopolysaccharides were not involved in its attachment, suggesting a predominant role by some other wall constituent, e.g. lipid. However, the more likely alternative to this

is that the attachment interaction was close and difficult to disrupt, perhaps offering configurational protection from the denaturing effects. It is important to consider the possibility that other protein degrading agents may, indeed, cause detachment of this species. The particular configuration adopted by cell surface proteins interacting with a substratum may cause differences in the efficiency of degradative enzymes. This may also be an alternative explanation for apparent differences in interaction between TCD and PD surfaces since molecular configurations at the two surfaces may differ considerably. Thus it is dangerous to entirely ignore the possible influence of wall proteins on bacterial attachment in general and in the particular case of the Flexibacter sp. A marine Pseudomonas sp, was detached from glass slides by trypsin, but not by pepsin, lysozyme or pronase, underlining the problem described above (Corpe, 1974b).

A variety of effects have been demonstrated by degradative enzymes on bacterial detachment from surfaces. Pronase and trypsin did not remove a Pseudomonas sp, from glass (Fletcher, 1980a), however, pronase caused detachment from PD and TCD at 20-25°C of the same bacterium (Fletcher & Marshall, 1982). It was also found that pronase varied in its effects at 15°C with TCD substratum. Trypsin consistently caused no detachment (Fletcher & Marshall, 1982). Danielsson et al. (1977) found that some strains of marine pseudomonads detached in the presence of pronase and trypsin. Gram-positive organisms have also been shown to detach in the presence of enzymes. Staphylococcus epidermidis significantly desorbed from FEP-teflon and cellulose acetate in the presence of pepsin (Hogt et al. 1982).

Periodate treatment has also been shown to remove a range of bacteria from solid surfaces. Though apparently in some instances pre-treatment with pyrophosphate was required before periodate treatment

successfully desorbed cells (Marshall, 1974). This again indicates the possibility of different configurations of cell surface molecules for the attached bacteria. Exo-polysaccharides may be involved in attachment even if there was no apparent desorption of cells in the case of the Flexibacter sp, or the Pseudomonas sp. However, periodate treatments have successfully detached Flexibacter aurantiacus CW7 and Hyphomicrobium vulgare ZV580 (Marshall & Cruickshank, 1973) and marine pseudomonads (Fletcher, 1980a ;Corpe, 1974) indicating the importance of polysaccharide exopolymers.

The results described above indicate roles for both protein and exopolysaccharide molecules at cell surfaces in bacterial attachment. The polysaccharide moieties will contribute to polar interactions while the proteins may contribute to both polar and non-polar interactions with the solid substrata. Not only will their relative importance in attachment interactions vary with the nature of the bacterial surface, but also with the type of interactions possible by the solid substratum. Thus, as indicated above the effect of protein and polysaccharide degradation will vary with bacterium and substratum.

7.5.5 pH and the Attachment of a FLEXIBACTER sp and three Non-Gliding Bacteria

The pH of the liquid phase had a major effect on the attachment of the four species investigated. The three non-gliding bacteria showed peaks in attachment at pH 5.5 to pH 7 while the attachment of the gliding bacterium decreased with increasing pH. This effect was found on both PD and TCD surfaces. Other workers have found that pH influences bacterial attachment to a variety of surfaces. Daniels (1980) indicated in a review of bacterial adsorption that the strongest attachment to soils, clays, hydrous metal oxides and ion exchange resins is often between pH 3 and 6. The attachment of several bacterial

species to starch was found to be maximum at pH 6 to 7, declining up to pH 10 when only 4% of the total cells would attach (Minato & Suto, 1976).

pH in the liquid bulk phase will affect both the solid substratum and the bacterium. Depending on the pH of a solution the surface potential changes, with changes in dissociation, and thus the extent of the double layer for the surface will vary. The effect will change with the solid substratum, and will influence physico-chemical interactions.

At low pH levels hydrogen ions might concentrate at a negatively charged surface. Adsorption of H^+ at the interface might result in the pH at a solid surface being different than that of the bulk liquid phase. This would consequently affect bacterial physiological processes, e.g. enzyme reactions which have pH optima. Hattori and Furusaka, (1961) found that the activity of Azotobacter agile on an ion exchange resin showed shifts in pH/activity curves to the alkaline side by one unit compared to unattached cells. Similar observations were made for E. coli (Hattori & Furusaka, 1960).

The influence of pH on bacteria has two facets: the first is the physico-chemical changes in the surface; the second is physiological. The peaks in attachment shown by the three non-gliding bacteria are related to some degree with pH conditions supporting maximum growth. However, good growth was shown over a far wider range than the attachment peaks and it is doubtful that over pH 5.5 to pH 8 that physiological factors dominate the interactions. This view is supported by the evidence from the growth rate experiments (Chapter 4) and growth phase experiments (Tables 7.1-7.4) which indicate no significant role of physiological activity in the attachment of these three species. However, below pH 5.5 and above pH 8 cellular activity may be so undermined as to have an unfavourable effect on attachment possibly by

altering cell surface characteristics. The attachment of the Flexibacter sp, bears no relation to its growth at the various pH levels. This is surprising since activity seems important in the attachment of this species. Such a result may indicate the prior accumulation of a constituent supporting attachment.

Changes in cell surface characteristics with changes in pH of the bulk liquid phase have been well demonstrated, usually by the electrophoretic mobility of bacteria, at different pH's (Marshall, 1967; Plummer & James, 1961; Marshall & Cruickshank, 1973). Bacterial species have been found to differ in their electrophoretic mobility with changes in pH. This was interpreted by Marshall (1967) and since by other workers, as the result of the different proportions of ionogenic groups on the bacterial surfaces and their different dissociation points.

Marshall considered the balance of ionization of carboxyl groups, which occurs particularly at alkaline pH's and confers a net negative charge on the cell, and amino groups, which occurs in acid conditions and results in a net positive cell charge, confers the characteristic charge to bacterial surfaces at a given pH. Sulphate and phosphate groups may react in a similar way to carboxyl groups, however, their dissociation constants are different. Thus the type of wall components, their proportions and dissociation constants will all effect the bacterial surface charge at a given pH. Burns (1979) suggested that the isoelectric point for most bacteria is pH 2 to pH 3 below which they will be positively charged..

The proportions of positively charged groups and negatively charged groups on the cell surface of four bacterial species investigated above have been shown to vary with growth rate (Chapter 4) and undoubtedly change with growth conditions. However, all four have been demonstrated

to possess both positive and negatively charged groups. Thus, with shifts in pH different numbers of carboxyl and amino groups will be ionized. The lower the pH the more positively charged groups on the surface; the higher the pH the more negatively charged groups. Since the polystyrene surfaces bear a predominately negative charge (Fletcher, 1976) TCD more than the PD surface, it might be expected that attachment would increase at lower pH's since more base groups and fewer acid groups would be ionized. This, however, does not occur with any but the Flexibacter sp, and the peaks in attachment for the other three species seem to reflect a balance in the charged groups. Changes in bacterial charge characteristics will also influence other phenomena such as double layer effects which in turn might influence attachment. However, the pH of the surrounding solution may not only affect the charge of the cell surface, but may also affect the three-dimensional configuration of the bacterial surface molecules by changing the ionization of groups within the molecule. This in turn may influence bacterial attachment to the solid surfaces by altering groups available for attachment interactions.

Thus, the effect of pH on bacterial attachment is varied and may represent a balance of all the forces described above. For the three non-gliding bacteria, pH's most suitable for attachment are at or just below neutrality, perhaps indicating large changes in cell surface characteristics in either alkaline or acid conditions. It is possible that a balance of negative and positive charged groups increases the number of potential types of interactions with the solid surface. The attachment of the gliding bacterium was highest at the lowest pH, possibly because of large numbers of positively charged groups and fewer negatively charged groups, or because the molecular surface structure was most stable at this pH and the conformation favoured attachment.

7.5.6 Temperature and the Attachment of a Gliding and three Non-Gliding Bacteria

The effect of temperature on the attachment of the gliding bacterium was considerable, a decline in attachment to both surfaces was associated with increasing temperature. The three non-gliding bacteria, on the other hand, had peaks in attachment between 20° and 30°. Thus the permanent attachment of the gliding and non-gliding bacteria differed considerably in response to temperature. Several workers have demonstrated that temperature has a large influence on levels of bacterial attachment. Such diverse bacterial species as Streptococcus faecium (Orstavik, 1977) and a marine Pseudomonas sp, (Fletcher, 1977) have been shown to increase their adhesion with increases in temperature. However, this has been limited to a fairly narrow range of experimental temperatures. Minato and Suto (1976) found that a mixed community of rumen bacteria were capable of attaching to starch over a range of temperatures from 4° to 40°. In general they found that there was little attachment below 20°C and maximum adhesion at 38°C. In further work (Minato & Suto, 1979) isolating individual species and strains of bacteria, predominantly Bacteroides species, they showed that all the bacteria attached well at 38°C, but some were incapable of attaching at 4°C. This indicated that individual species respond differently to temperature effects and may suggest separate attachment mechanisms.

The influence of temperature on the mechanisms of bacterial attachment are complicated. As with pH, the effects can be divided into two categories: first, on bacterial physiology; second, on the physico-chemical attachment interactions. The role played by temperature in bacterial physiology is complex and, apart from its obvious influence on reaction rates, may be varied. However, increases

in bacterial growth and activity over the range of temperatures examined above do not indicate a close relationship between bacterial physiological activity and attachment. As with pH the effect of temperature on the attachment of the Flexibacter sp, was not necessarily related to physiological activity.

Temperature has a series of different influences on the physico-chemical parameters of adhesion. Bacteria can be regarded as colloidal sized particles (Marshall, 1976) and will, therefore, be in kinetic equilibrium with the molecules of the surrounding medium. Thermal movements of the latter result in Brownian motion which is directly related to temperature.

$$D = RT/6\pi n a N$$

where D is the diffusion coefficient, R is the gas constant, T is the absolute temperature, N is Avagadro's number, n the viscosity of the medium and a the radius of the colloidal particle. Thus, as temperature increases, which also results in a decrease in medium viscosity, Brownian motion will increase. This in turn will increase the probability of bacterial contact with the solid substratum concomitant with the increase in random motion. However, none of the bacteria shows a continuing increase in adhesion with temperature indicating that other phenomena dominate the interaction.

Lower temperatures will cause the viscosity of the medium to increase which in turn will tend to act against bacterial attachment. This may be a partial explanation for the lower attachment of the three non-gliding bacteria which decreases to variable extents at the lower temperatures. However, an increase in the viscosity of an adhesive may promote attachment, this may be the case for the Flexibacter sp. A further influence of temperature is in the balance between physical and chemical adsorption, the relationship is complex depending, basically,

on whether the adsorption process is endothermic or exothermic (Kipling, 1965). Generally, lower temperatures favour physical adsorption while chemisorption is favoured by higher temperatures, though certain types of physical adsorption can be increased at higher temperatures, e.g. hydrophobic bonding (Shaw, 1970; Ben-Nain, 1980). Physical adsorption involves attractive forces such as London-van der Waals forces, electrostatic and hydrogen bonding etc, which are relatively non-specific, while chemiadsorption is normally a stronger, more selective interaction. It is, however, unlikely that chemiadsorption is significantly involved in the attachment interactions of these bacteria since it describes chemical reactions. Physical adsorption will certainly be the more important of the two phenomena. The peaks in attachment for the three non-gliding bacteria between 20-25°C may represent the temperatures at which the cell surface configurations of the bacteria favour attachment, rather than just changes in physical adsorption phenomena. One feature which should not be ignored is that the temperature of the cell surface itself may influence its molecular configurations and, thereby, influence the cells subsequent attachment.

The Flexibacter sp, was unique among the bacteria investigated, in that attachment was maximum at the lowest temperatures, particularly to the PD surface. As described above, the Flexibacter sp, has hydrophobic poles to its cell. If this were a crucial feature in the attachment of this species, attachment would be expected to increase with temperature since hydrophobic interactions are favoured by increasing temperature (Ben-Nain, 1980). Fletcher and Marshall (1982) found that attachment of a marine pseudomonad to a PD surface decreased more than that to a TCD surface at 15°C compared to 25°C. They suggested that since PD was the more hydrophobic surface the greater effect of decreasing temperatures was due to the predominance of hydrophobic interactions. Although

hydrophobic interactions between the solid surface and the bacterial surface may well occur, the effects of temperature on all the species investigated above do not indicate that they are of predominant importance. Apparently the hydrophobic portion of the Flexibacter sp, is not the prime determinant of attachment.

As indicated above, part of the role played by the activity of the Flexibacter sp, in attachment might be the accumulation of a material, probably polymeric, which supports and increases the attachment of this bacterium. The effect of temperature on the Flexibacter sp, adhesion to solid surfaces might well support this view. The strength of adhesive polymers has been shown to decrease with increases in temperature due to viscosity changes (Stanley & Rose, 1967) and it is this feature that may cause the apparent inverse relationship between attachment and temperature.

Thus the mechanisms and determinations in the attachment of the three non-gliding bacteria and the gliding bacterium appears to be very different. The adhesion of the non-gliding bacteria is probably purely the result of physico-chemical interactions, the balance of which vary with environmental conditions and the nature of the cell surface. The Flexibacter sp, on the other hand, presents several unique characteristics which may be the result of adaptations to the necessity to approach and attach to a substratum in order to reap benefits from its gliding motility.

The hydrophobic portions of the Flexibacter cell may encourage approach to a surface and to some extent association with that surface. However, it does not apparently represent a major determinant of levels of attachment which seem closely linked to bacterial activity. This link between the activity of the Flexibacter sp, and its attachment may be related to increased production of material supportive of attachment,

i.e. an exopolymer. Such polymeric material which has been observed to be produced by some Flexibacter sp (Marshall & Cruickshank, 1973; Ridgway, 1977) may be the major influence on the permanent attachment of these species. The nature of the interaction between substratum and the Flexibacter surface was consistently affected by the presence of different molecular types in the suspending medium. Attachment was always lowered by the presence of media (Chapter 6). This may indicate that this bacterium's attachment interaction with a surface can be rapidly inhibited possibly by disrupting polymeric binding capacities so that its adhesive capabilities were lowered.

7.5.7 The Role of Exopolymer in the Permanent and Temporary Attachment of the FLEXIBACTER sp

There are many different models for the mechanism of gliding motility, however, polymeric material has been implicated as a major component of the mechanism (Humphrey et al. 1979; Duxbury et al. 1980). Arlauskas and Burchard (1982) found that gliding motility of Flexibacter FS-1 could be promoted by the presence on surfaces of several different polysaccharides, e.g. pectin and polygalacturonic acid, which were known to exhibit the properties of temporary adhesives. A water soluble slime consisting of a glycoprotein, and demonstrating the characteristics of a linear colloid, was isolated from Flexibacter BH3 (Humphrey et al. 1979). These workers suggested that this extracellular slime produced an environment of increased adhesiveness for the bacterium to the substratum while allowing translational motion across the surface. It provided the functions of a temporary Stefan adhesive, i.e. an adhesive interaction where the force required for separation of the surfaces is far greater than the horizontal drag (Crisp, 1973). Thus the bacterium is able to move by gliding across a surface while remaining attached to that surface.

It is clear for the Flexibacter sp. investigated here, that nutrient concentration has a large effect on gliding, which was apparently limited to fairly low nutrient conditions (Table 7.10).

There was also an inverse relationship between the extent of polymer production and gliding motility. Thus as polymer production increased with increasing nutrient concentration so gliding motility decreased. Duxbury et al (1980) also found a link between gliding motility and nutrient concentration, but the effect was caused by increasing casamino acid concentrations inhibiting spreading growth on agar surfaces rather than increases in glucose concentrations.

There are two possible explanations for the apparent inhibition of gliding motility by polymer production. Humphrey et al. (1979) indicated that the viscosity of the slime increased rapidly as its concentration increased, a characteristic of linear colloids. As viscosity increases so both the force preventing separation and the horizontal drag will increase. There may be a critical point in the accumulation of polymer between the bacterial surface and the solid substratum after which the magnitude of horizontal drag becomes so large that the bacterium cannot energetically overcome it. Thus the cell is no longer capable of gliding motility, but is permanently attached.

The second possibility is that two distinct types of polymers are produced by this Flexibacter sp one producing the effects of a temporary adhesive, the second the effects of a permanent adhesive. The increases in nutrient concentrations may cause increased production of the permanent polymeric adhesive over the temporary adhesive, thus inhibiting gliding motility. It was interesting that growth on sucrose medium caused the production of little polymer, but no concomitant increase in gliding motility, possibly indicating the production of both polymeric materials was inhibited. Humphrey et al. (1979) isolated two extracellular fractions from Flexibacter BH3 the first, the glycoprotein slime, the proposed temporary adhesive, the second an insoluble fraction consisting of lipopolysaccharide. They were unsure whether this latter fraction was involved in gliding motility. It may be such material which contributes to permanent attachment and eventually to the

inhibition of gliding motility.

The effect of nutrient concentrations on the gliding of the Flexibacter sp, described in this Chapter and by other workers (Stainer, 1947) for different gliding bacteria, may indicate an interesting ecological adaptation. The presence of high concentrations of nutrients apparently inhibits motility, and thus bacteria will remain stationary in a favourable nutrient-sufficient environment. As growth and utilization of the substrate proceeds so nutrient levels in the immediate vicinity may become depleted. When nutrient levels become sufficiently low, gliding motility is no longer blocked and the Flexibacter sp, moves across the interface until a suitable environment of higher substrate concentration is reached. Interestingly, Duxbury et al. (1980) found that Flexibacter BH3 could maintain gliding motility in the absence of exogenous substrate for up to twenty hours.

There may be an alternation between permanent and temporary adhesion by the Flexibacter sp on a solid substratum. The initial interaction with a solid substratum may be permanent in nature remaining as such or changing to temporary, depending on the micro-environmental conditions at the interface.

It is apparent from the data in this Chapter and previous Chapters that there is a danger of generalizing on the mechanisms of bacterial attachment. The Flexibacter sp, showed very different attachment interactions with solid surfaces than any of the three non-gliding bacteria. This possibly indicates a degree of adaptation to solid surfaces on the part of this bacterium. Clearly interactions with solid surfaces may change with bacterium depending on the special characteristics and requirements of the individual species.

7.6 SUMMARY

- 1) The attachment of the Flexibacter sp, and the three non-gliding bacteria increased with cell concentration until a plateau level was reached. The rate of increase and the level of the plateau was

different for each species and for each substratum. The increases in attachment were probably caused by a larger opportunity for contacts, i.e. collisions, and therefore attachment interactions between the bacteria and surface the higher the cell concentration. The different plateau levels may relate to the maximum adsorption capacity of the surfaces for the bacteria.

2) P. fluorescens, E. cloacae and the Chromobacterium sp, showed a variation in attachment levels with growth phase in batch culture, which varied with substratum. These differences were probably related to changes in cell surface characteristics. The gliding bacterium Flexibacter sp, showed an increase in attachment with stationary phase cells but a significant decrease in death phase. This, linked with an increase in attachment with increased activity on the part of this bacterium, may indicate that attachment might be partially an active process for the Flexibacter sp. This may be due to the production of a polymeric material which promotes attachment, or a more immediate response on contact with a substratum.

3) The Flexibacter sp, orientated perpendicularly to an oil/water interface, none of the non-gliding bacteria associated with the interface in this manner. The orientation suggests that the poles of the Flexibacter cell were hydrophobic in nature.

4) Periodate and protease treatment of attached cells demonstrated that both protein and polysaccharide components of the cell wall of the three non-gliding bacteria could be involved in their attachment. The contribution made by each component varied with bacterium and solid surface. The Flexibacter sp. did not detach from the surface in the presence of either periodate or protease. This could suggest that neither proteins nor polysaccharides were the prime surface components involved in attachment, a more likely explanation that binding to the substratum was very close allowing neither of these degradative

components access to the attachment sites. The presence of chloramphenicol caused no detachment of any of the bacterial species indicating that protein synthesis was not a requirement for maintaining attachment.

5) The attachment responses of the three non-gliding bacteria and the gliding bacterium to changes in pH were different. The Flexibacter sp, decreased adhesion with increasing pH, while the other three species showed peaks in attachment to both surfaces between pH 5.5 and 7. These attachment profiles were not apparently closely related to bacterial physiology. The predominant influence of pH was probably the ionization of carboxyl and amino groups on the bacterial surface and changes in the configuration of cell surface molecules. This will affect the physico-chemical interaction with the solid surface, which will itself present changed conditions to approaching bacteria. The attachment profiles of all four of the organisms probably represent the areas of most favourable surface charge and configuration to enter into a range of binding interactions with a solid surface.

6) The attachment temperature again had a different effect on the three non-gliding bacteria and the gliding bacterium. The Flexibacter sp, showed a decline in attachment with increasing temperature, while the other three species had peaks in adhesion between 20° and 30°C. Bacterial physiology did not appear to play a direct role, nor did levels of motility. Brownian motion, and therefore contacts with the substratum, increased with temperature but this was not related to increased attachment for any of the bacteria. Since it is unlikely that chemiadsorption is involved in bacterial attachment and since physical adsorption is favoured by lower temperatures, the peaks in attachment are probably due to favourable molecular configurations on the bacterial cell surfaces (which may be altered with temperature).

Although the Flexibacter sp, had distinct hydrophobic portions to

the cell, hydrophobic interactions did not predominate in its adhesion interactions, e.g. attachment decreased with increasing temperature, while hydrophobic interactions increase with temperature. Since the strength of adhesive polymers decrease with increases in temperature, as did the Flexibacter species attachment, this may be a further indication of the role of adhesive polymers in the attachment of this organism. An effect of polymeric adhesive strength may represent the more probable influence of temperature on the attachment of the Flexibacter sp.

7) As nutrient concentration increased so the gliding motility of the Flexibacter sp, was inhibited on solid media, and large amounts of polymer were produced. There was an inverse relationship between polymer production and gliding motility. A temporary adhesive would be required for gliding. Inhibition of gliding motility may have occurred either when the concentration of temporary adhesive polymer became so high that viscosity effects caused the horizontal drag on the bacterium to become so large that it could not be energetically overcome; or if two different types of polymer were produced at different nutrient concentrations, i.e. at low nutrient levels a temporary adhesive, whereas at high levels, a permanent adhesive.

The effect of nutrients on the gliding motility of this Flexibacter sp, may represent an adaptive advantage, since it may encourage gliding motility when nutrients become limiting and a form of permanent adhesion in advantageous conditions.

8) There were major differences in the attachment responses of P. fluorescens, E. cloacae, the Chromobacterium sp, and the Flexibacter sp, to solid substrata. Clearly, it is difficult to generalize on bacterial attachment mechanisms. The Flexibacter sp, may be more specialized in its interactions with solid surfaces due to its ability to demonstrate gliding motility.

CHAPTER EIGHT

FINAL DISCUSSION AND CONCLUSIONS8.1 Factors Affecting Bacterial Attachment to Solid Surfaces

Bacterial attachment to solid surfaces in aqueous systems is a complex tripartite interaction between the bacterium, the solid substratum and the liquid phase. The evidence overwhelmingly suggests that bacterial adhesion is primarily the result of physico-chemical interactions between these three components, and further that they are influenced by changes in environmental conditions (Fig. 8.1).

The range of variables that can influence these physico-chemical interactions is immense, and possibly quite small changes in any one variable might cause large differences in bacterial attachment levels and/or rates of attachment (Table 8.1). Microbial factors are initially dominated by the genetic make-up of the bacterium, which will determine such features as the constitution of the cell wall, polymer production and the possibility of an active response to an association with a substratum. The Flexibacter sp, investigated in this work clearly shows a different type of interaction with solid substrata than any of the other bacteria, and may have an attachment mechanism linked to activity, i.e. possibly involving the production of a polymeric material. The cell wall of the Flexibacter sp, apparently possesses hydrophobic areas, specifically at the poles of the cell, this must require considerable genetic control. The gliding motility and possible specialized attachment mechanisms, e.g. hydrophobic poles, a link between attachment and bacterial activity, may represent a more highly refined adaptation to 'surface life' than found in the non-gliding permanently attached bacteria.

The cell surface characteristics of bacteria are not only genetically controlled, but are further influenced by a range of

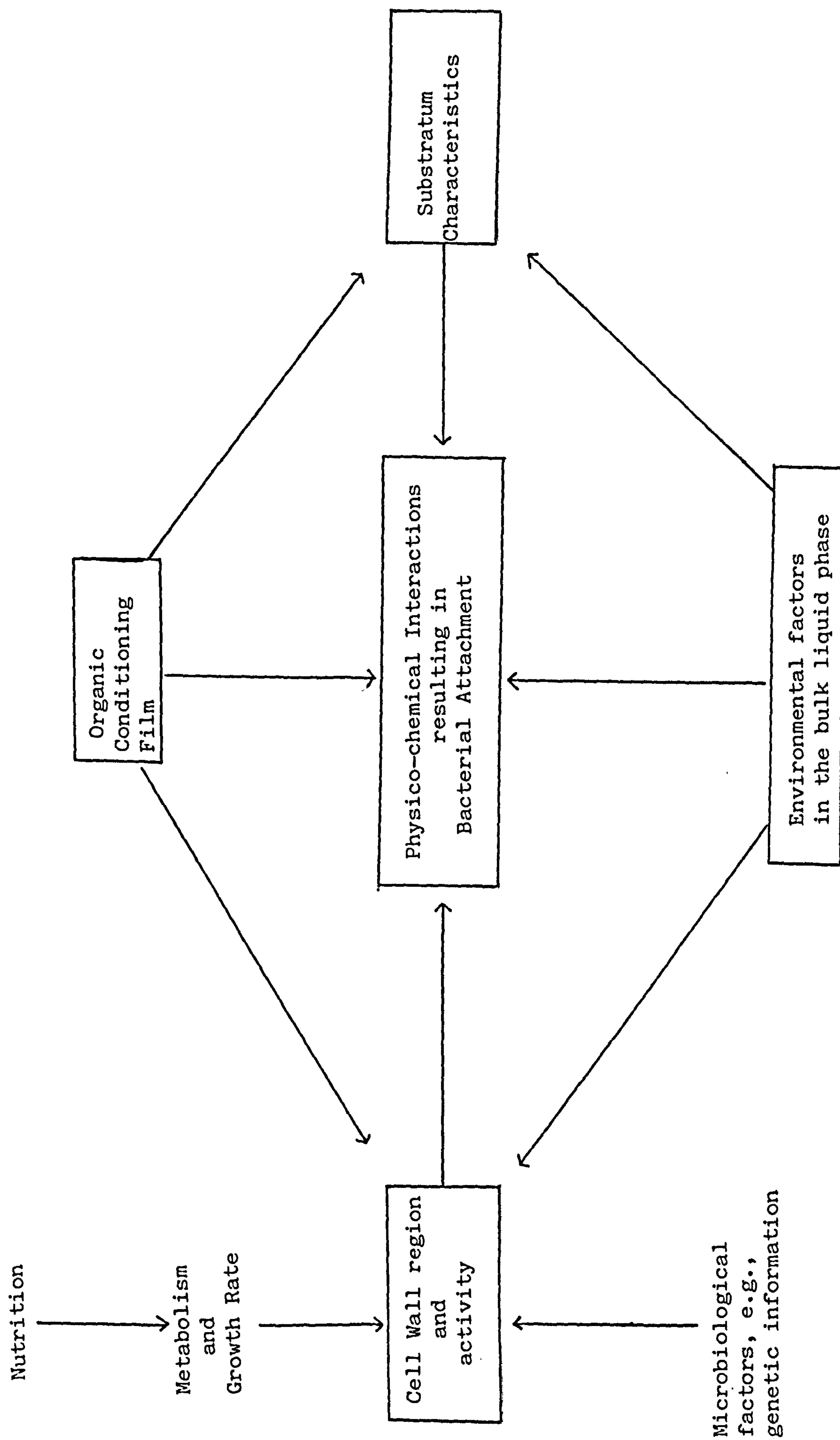


FIG. 8.1 Schematic Representation of Factors Affecting Bacterial Attachment to Solid Surfaces

TABLE 8.1 Factors Affecting Bacterial Attachment to Solid Surfaces

MICROBIAL FACTORS	SUBSTRATUM FACTORS	ENVIRONMENTAL FACTORS	BIOLOGICAL FACTORS	PHYSICO-CHEMICAL INTERACTIONS
Genetic	Surface chemistry (bonding type)	pH	Inoculum size	DLVO effects
Cell surface	Surface texture	ionic strength	Incubation time	Surface tension effects
Nutrition	Surface tension (bonding capacity)	temperature	presence of other organisms or strains	Hydrophobic inter- actions
Growth Rate and activity	Conditioning film	C/N ratio	competition between organisms	Change interactions e.g. ionic bonding dipole-dipole interactions, H-bonding etc
Physiological age		carbon source		
		nitrogen source		
		aeration		Steric exclusion
Motility				Polymer bridging
				Hydration effects
				Viscosity effects

environmental factors, including C/N ratio and carbon source. A lasting impression of the results presented during this work is the variability of bacterial cell surfaces and the magnitude of the effect this has on subsequent bacterial attachment. Although the extent of these effects varies with bacterial species, the changes are not only the result of nutrition but also relate to changes in bacterial growth rate and physiological age. Further modifications in bacterial cell surface characteristics during growth may also occur with changes in conditions such as pH and temperature. Add to this indirect influence on bacterial surfaces those more direct physico-chemical effects caused by environmental conditions (e.g. ions binding to cell surfaces; ionization of different cell surface groups at different pH; temperature effects) the possible flexibility and variability within bacterial attachment systems becomes enormous. This has, to some extent, been demonstrated in Chapters 4 to 7.

Not only is the bacterium prone to change, but solid substrata, already exerting individual influences on attachment, will be modified by environmental conditions. The most spectacular, and probably the dominant, influence on solid surfaces will be the development of conditioning films - well documented for natural aquatic habitats. Such organic layers at the surface will mediate attachment interactions between the substratum and bacterium, and may promote or inhibit adhesion. It should not be overlooked that the bacteria will probably, also have an organic conditioning layer associated with their surfaces, further affecting attachment.

The final determinant of bacterial attachment to a solid surface will be the balance of physico-chemical forces for and against adhesion. There is a large range of physico-chemical interactions which may influence bacterial adsorption processes (Table 8.1), and it is probable

that the process is a multifactorial interaction of several different types with no one interaction dominating.

The importance of individual physico-chemical factors will vary with the bacterial cell surface, the solid substratum and environmental factors, thus the balance of forces may not be identical in any two attachment interactions. It is, therefore, extremely unlikely that particular types of interactions e.g. hydrophobic bonding, if a dominant participant in certain attachment processes, will be so for all bacterial attachments in all conditions. Hydrophobic bonding, although playing a role in the various bacterial attachments investigated was not the prime determinant of this association, even in the case of the Flexibacter sp, which had highly hydrophobic polar regions on its cell surface.

Accurate description of attachment processes for individual bacteria is fraught with difficulties. In discussions on bacterial adsorption phenomena it is essential to describe the microorganism involved. Certain groups of bacteria in aquatic habitats may comply to particular attachment criteria. Thus copiotrophic non-gliding bacteria which undergo permanent attachment may be categorised together, e.g. P. fluorescens, E. cloacae, and the Chromobacterium sp described above, and may largely have no direct control on their attachment, rather an indirect influence through changes with cell surface characteristics. On the other hand, bacteria possessing a degree of adaptation to solid surfaces, e.g. copiotrophic gliding bacteria may be classified in another group possibly actively involved in their attachment to solid substrata e.g. the Flexibacter sp, described above. However, overall generalizations on attachment mechanisms may be dangerous and further investigations over a broader range of species is essential.

8.2 The Role of Exopolymeric Material in Bacterial Attachment

Bacterial attachment to solid surfaces has often been considered to be mediated or controlled by the production of extracellular polymeric material. In one of the earliest observations on periphytic bacteria, ZoBell (1943) suggested that on contact with a substratum bacteria secrete a 'cementing' substance which effectively mediates attachment. Such a view has been supported by subsequent workers (Corpe, 1974a ; Costerton et al, 1978) who have further proposed that polyanionic carbohydrate polymers may participate in initial interactions between bacteria and solid surfaces.

Corpe (1970b) demonstrated that many different marine periphytic bacteria were capable of producing polymeric material, specifically polyanions, though in variable quantities. Indeed, there can be no dispute that surface associated bacteria often produce copious amounts of extracellular material which eventually embeds the attached bacteria in a polymeric matrix. This has been shown for a variety of situations, from mountain streams (Geesey et al, 1977) and marine habitats (Dempsey, 1981) to laboratory conditions (Wardell et al, 1980; Marshall et al, 1971a; Fletcher & Floodgate, 1973). However, although production of extracellular material often dominates such associations it is by no means universal in its presence. Some firmly attached bacteria produce no, or insignificant quantities of polymeric material (Dempsey, 1981; Marshall et al, 1971a) and still remain permanently attached.

A survey of the literature examining conditions promoting the production of exopolymers by bacteria (Chapter 4) indicates a wide variation in the extent of biosynthesis with bacterial species, growth conditions and growth phase etc. Thus the production of extracellular material is very dependent on surrounding environmental factors, and it

is this dependence which has been suggested as determining the presence of polymer around attached bacteria. It has often been found that periphytic bacteria lacking polymer are present in carbon-limiting conditions, which restricts polymeric biosynthesis (Wardell et al, 1980; Brown et al, 1977; Dempsey, 1981). In natural conditions different nutrient levels have been clearly shown to result in different quantities of polymeric material on solid surfaces. Inorganic detritus in an oligotrophic lake was associated with little polymeric substances, while detritus from a nutritively rich aquatic habitat was extensively covered by exopolymers (Paerl et al, 1975; cited in Paerl, 1980). This variability in extracellular polyanionic polysaccharide production draws into question the applicability of a system so prone to environmental oscillations actually fulfilling a role as an active determinant of attachment. Further, permanent irreversible bacterial attachment does occur in its absence.

Recent evidence has, in fact, indicated that the biosynthesis of large amounts of extracellular polysaccharide may actually inhibit the primary stages of bacterial attachment. Using enrichment chemostats Brown et al (1977) found that carbon-limited cultures produced a diverse surface-associated microbial community, the permanent attachment of which was not dependent on the presence of copious amounts of expolysaccharide. Nitrogen-limited cultures, however, had surfaces coated with large quantities of polymeric material but few bacteria were attached. Similar trends were later found by Wardell et al (1980) in enrichments of different organisms. In a comparison of attachment between a capsulated Staphylococcus saprophyticus and a non-capsulated Staphylococcus epidermidis to solid surfaces, the attachment of the capsulated species was found to be less (Hogt et al, 1982). It was clearly and convincingly demonstrated that mucoid mutants of a

Pseudomonas fluorescens showed low levels of attachment to PD and TCD surfaces compared to a wild-type and a mutant which produced little extracellular polysaccharide (Pringle et al, 1983). Thus, extracellular polysaccharide material, thought to act as cellular adhesives, can actually prohibit attachment.

From physico-chemical considerations it is not difficult to predict, theoretically, that exopolysaccharides might inhibit bacterial attachment. Steric exclusion parameters (described in Chapter 6) alone predict that a polymeric matrix around bacteria may inhibit attachment (Maroudas, 1975b) and ionic repression may also play a role (Pethica, 1961). Pringle et al (1983) also suggest that the exopolymers might inhibit attachment by saturating binding sites on a solid surface.

Floodgate (1972) suggested that the initial phases of attachment were dictated by physico-chemical forces (Chapter 5) and that the production of polymeric adhesives represented a second phase in the process of surface colonization. Similarly, Pringle et al (1983) concluded that acidic polysaccharide materials were not involved in primary attachment but represented a time dependent biosynthesis process. These views are supported by observations that polymer often develops in later stages of bacterial film development (DiSalvo & Daniels, 1975). The implication then, is that initial stages of bacterial attachment are governed by physico-chemical interactions between bacterial surfaces and solid surfaces, only involving bacterial exopolymers as far as they affect the adsorption interactions.

Variability in bacterial surface characteristics appear to affect bacterial attachment to substrata (Chapter 4) and this probably involves a range of surface molecular types. The presence of polymeric material acts as another component in the balance of physico-chemical forces favouring or inhibiting attachment of bacteria to solid surfaces.

Exopolymers may be predominantly inhibitory to bacterial adhesion, however, as pointed out above, it is dangerous to generalize and there may be instances where they favour and encourage attachment.

The role of exopolymers in attachment might vary from species to species. Staphylococcus salivarius which on electron microscopic examination possessed a polymeric coat, reduced levels of attachment to glass and polystyrene when the polymeric layer was removed by trypsin treatment, while the attachment of S. mitior, which produced a thinner 'fuzzy' coat, was less affected (Rutter & Abbott, 1978). It was suggested that the polymeric layer might produce polymer bridging effects, which have frequently been demonstrated as important in bacterial flocculation, or may produce favourable charge distributions for bacterial attachment (Rutter & Abbott, 1978). The initial indications in the attachment of the Flexibacter sp, investigated above, suggest that there may be an adhesive contribution made by an exocellular constituent, possibly polymeric. Although further work is required to fully substantiate such a possibility, it cannot be ignored. However, the influence of exopolymers will always be governed by physico-chemical forces and the outcome of the interaction may well vary with the solid surface, the nature of the conditioning film and the environmental conditions in the bulk liquid phase.

The concept of polymeric material functioning as a secondary adhesive in bacterial attachment may be questionable for several reasons. First, bacteria have been shown to remain attached to solid surfaces for long periods of time (days) even in the absence of visible polymeric material (Pringle et al, 1983; Dempsey, 1981). Second, the production of such extracellular material differs so much between culture conditions and with bacterial species (Chapter 4) that its production could not be guaranteed for each attachment situation. Last,

for polymers to act as effective adhesives it may not be necessary for the production of the copious quantities as found, for example, in nitrogen-deficient environments. A narrow band between cell and substratum would be a sufficient bridging adhesive and need only be maintained. The production of large amounts of exopolysaccharides would seem energetically wasteful and an inappropriate use of nutrients. Further, such elaborate polymeric systems eventually present the now immobile permanently attached bacteria with apparently insurmountable problems. When the bacterial film thickness becomes sufficient penetration of oxygen, and nutrients by diffusion will be limited (Atkinson & Fowler, 1974; Dudman, 1977) and below a certain depth bacteria will be presented with anaerobic perhaps nutrient deficient conditions, which they cannot escape. It may be in natural conditions films do not achieve such depth due to grazing by protozoa or nutrient limitation, but such problems frequently occur in fermenter and waste treatment situations.

The production of exopolysaccharides by free-living bacteria has long been documented (Wilkinson, 1958). Their frequent occurrence in natural conditions has led to the conclusion that they confer a growth or survival advantage to bacteria. Many different protective functions have been proposed and experimentally supported (Wilkinson, 1958; Dudman, 1977; Costerton et al, 1974; Costerton et al, 1981). It has further been suggested that they may modulate the environment immediately surrounding the bacteria (Dudman, 1977; Costerton et al, 1974). Functions such as protection from phagocytosis; bactericidal agents; bacteriophages; predation; dessication have all been suggested and have largely been substantiated for an exopolymeric layer. Such surface materials may also control the bacterium's immediate environment by acting as a general physico-chemical barrier (Dudman,

1977) as metallic ions may be immobilized in the matrix (Corpe, 1975), or the polysaccharides may bind certain other ions or perhaps limiting nutrients. Thus the production of exopolymers in free-living environments is probably favoured by conferring a survival and perhaps competitive advantage on the bacterium. If selection pressure is lost in laboratory conditions then many bacteria no longer produce exopolysaccharide (Wilkinson, 1958). Interestingly, Wilkinson (1958) suggested that the highly charged exopolysaccharide layer might actually aid in dispersal since it would tend to dissociate from like-charged surfaces.

All the protective and modulating functions of polymers described above will still be functional and advantageous to bacteria attached to surfaces. Indeed, the polymer might confer further advantages. A bacterium in a liquid bulk phase will exist in a three-dimensional medium, with ready diffusion of components to and from the bacterial surface. Costerton et al (1974) proposed that the cell wall can be considered in terms of a metabolically important functional organelle. The question arises as to the consequences to that organelle when it no longer exists in a three-dimensional environment, but is forced into the two-dimensional situation of attachment on a solid surface. It is possible that considerable disruption of its functions could result, not only due to direct physical consequences, e.g. disruption of proteins, but also because of the accumulation of ions etc. Thus the production of exopolymers might offer a further survival advantage to an attached bacterium by re-instating a three-dimensional environment.

Since the production of extracellular polymeric material is common for a variety of free-living bacteria which can become periphytic, its production by periphytic organisms is probably not stimulated by the

solid surface. It may be a normal biosynthetic product selected for environmentally since it confers adaptive advantages to the bacteria, the fact that it appears to support bacterial attachment to solid surfaces may be a secondary feature. Polymeric material may not be produced to aid bacterial adhesion but in the 'normal' course of events depending on nutrient concentrations etc. The presence of large amounts of polymeric material at a solid/liquid interface might be the result of physical changes in conditions between free-living and attached bacteria. The same quantity of polymer might be produced by both the free-living and periphytic bacteria, but in the case of the former the polymer is naturally released from the surface of the organism or removed by hydrodynamic forces and may require continuous replenishment. A periphytic bacterium will be in a very different position, any polymer it produces will probably be retained on the solid surface by adsorption and possibly will be slightly denatured. Thus the exopolymer may not be removed from the vicinity of the immobilized bacterium which may continue to produce polymer at normal rates. Large quantities of polymer would eventually accumulate possibly producing an unfavourable environment for the periphytic bacterium, and inhibiting the attachment of further bacteria.

8.3 The Role of Water in Bacterial Attachment

Bacterial cell surfaces are extensively hydrated and the water molecules associated with their surfaces may be in a highly ordered form. Similarly solid surfaces will structure water molecules, with one or two condensed physically bound layers of water. Attractive and repulsive interactions between a solid substratum and a bacterium may be induced by the ordered bound water at their surface. The importance of such an influence on bacterial attachment should never be underestimated, though experimentally it may be exceptionally difficult

to determine. Indeed, given the levels of hydration of bacterial surface layers, particularly polymeric layers, water might play a very major role in attachment interactions.

Depending on the particular surface, hydration forces have been found to dominate interactions below 3 nm. Such forces can be either attractive or repulsive depending on pH and cation concentration (Israelachuli & Adams, 1976). In mica-water-mica systems up to pH 4 no short range hydration repulsion was observed and surfaces were in primary minimum contact. However, with added electrolyte, hydrated cations replaced adsorbed H^+ ions, and induced water structure, causing strong repulsive hydration forces. This was dependent on the cation type (Israelachuli & Pashley, 1982), and was also observed for air bubbles and clay particles. In pure water, or dilute electrolyte solutions, hydration forces will probably only exist if the surfaces already contain hydrated ionic species (Israelachuli & Pashley, 1982). Such is the case for the bacterial surface layers and also a solid substratum when there is an organic conditioning film present on that surface; the organic molecules such as glycoproteins will be hydrated, i.e. associated with ordered water (Baier, 1980b; Lewin, 1974). Layers of polymer associated with attached cells may also inhibit further bacterial adhesion due to repulsive hydration forces.

If bacterial attachment caused the extrusion of ordered water into the random bulk water phase, then attachment would be energetically favoured due to increasing entropy, e.g. hydrophobic interactions would have this effect (Chapter 5). Lewin (1974) suggested that water molecules might be able to act as a bridge between two loci because of their dipolarity and combined H-- bonding and H-- acceptance capabilities. Thus, ordered water structure at interfaces may not necessarily result in repulsive hydration forces.

The role and importance of water molecules in bacterial attachment will undoubtedly vary with the bacterial surface, the solid surface and environmental conditions. During the normal staining procedures of the attachment assays described in previous Chapters, if at any time the PD-attached bacterium system was not maintained under a layer of water, then the bacteria would 'peel' from the surface in unison with the water. This did not occur from the TCD surface or if either surface was treated with a conditioning layer. The PD surface would be largely repulsive to water since it is a relatively hydrophobic surface, while TCD and an organic conditioning layer may have water more closely bound to them. Such an observation implies the significance of water in the bacterial attachment interaction and indicates its role may differ with solid substratum. Thus, the role played by water in bacterial attachment should not be underestimated and deserves investigation.

8.4 The Attachment of Mixed Bacterial Populations

In natural environments there will be a mixed bacterial community and this will exert an influence on levels of bacterial attachment. The presence of other bacterial species, both in the liquid bulk phase and attached to the surface, has been shown above, to affect the subsequent attachment of individual species within the community. The interactions are complex and may be altered by quite small changes in species numbers and composition (Chapter 3). The variations in attachment need not only be the result of changes in physico-chemical interactions but may also be the result of competition between bacteria.

In both freshwater and marine habitats then, there are potentially many factors which will influence biofilm development. The levels of individual bacteria attaching to solid substrata will be influenced by the solid substratum, the presence of other bacteria and environmental factors such as pH, temperature and nutritive status. These effects

will be direct on the physico-chemical interactions and indirect by affecting bacterial cell surface characteristics and thence attachment. The relative numbers of different bacterial species and strains attaching will, then, be variable and this initial species composition may have a lasting effect on the nature, activity and biochemical re-activity of the developing biofilm. Since attached films represent a major site of bacterial accumulation and, potentially, activity in natural habitats, such variations in biofilm characteristics might have a significant ecological impact on the surrounding environment. Further, in man-made systems where biofilms represent a significant biofouling hazard the effects and extent of the problem may well differ with environmental conditions and the nature of the developing film.

8.5 Suggestions for Future Work

The results presented in this thesis underline several areas of work worthy of further study. Five sections of future work can be envisaged:

- 1) Further analysis of the physico-chemical basis of bacterial attachment interactions with solid surfaces, with particular reference to different molecular types on the bacterial surface and their role in the attachment process.
- 2) Investigation into the role played by bacterial exopolymers in the permanent attachment of bacteria, whether they promote or inhibit the initial stages of copiotrophic bacterial attachment.
- 3) Analysis of the role of water molecules in attachment interactions.
- 4) Further examination of the attachment of bacterial communities and the effect of the whole community on the attachment of its individual members. This should involve an investigation into the type of interactions between species which affect their attachment, and an

analysis of the subsequent influence on biofilm development.

5) Investigation into the attachment of the Flexibacter sp, whether it is an active process and/or involves the production of an exopolymer. An analysis of the type of exopolymer produced should be made, particularly with respect to determining whether the Flexibacter sp, produces only one type of exopolymer or two, i.e. one mediating permanent attachment the other the temporary attachment necessary for gliding motility. Further work on this organism might study the effect of nutrients on gliding motility and permanent adhesion when attached to solid surfaces, and the role of exopolymer production on these processes.

Since copiotrophic bacteria represent the initial colonizers of solid surfaces in natural environments (Section 1.3) an understanding of the basis for their attachment is essential, and should involve both pure and mixed populations of bacteria.

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A P P E N D I C E S

APPENDIX

TABLE 1. GROWTH, MOTILITY AND GRAM STAIN CHARACTERISTICS OF SELECTED BACTERIA

Organism No.	Identification	Gram Stain	Cell Morphology	Motility Liquid Medium	Solid Surface Gliding	Aggregation in Broth culture	Pigments produced on PYE
H 3	Acinetobacter calcoaceticus	-	cocco/bacillus	-	-	-	-
H 7	Acinetobacter sp.	-	cocco/bacillus	-	-	-	-
H 20	Acinetobacter sp.	V	cocco/bacillus	-	-	-	-
H 34	Acinetobacter sp.	-	cocco/bacillus	-	-	-	-
H 42	Acinetobacter calcoaceticus	-	cocco/bacillus	-	-	-	-
H 22	Aeromonas hydrophila	-	bacillus	+	-	-	-
H 48	Aeromonas hydrophila	-	bacillus	+	-	-	-
H 31	Chromobacterium sp.	-	bacillus	+	-	+	purple
H 16	Coryneform sp.	+	bacillus	+	-	-	yellow
H 12	Enterobacter cloacae	-	bacillus	+	-	+	-
H 15	Pseudomonas aeruginosa	-	bacillus	+	-	-	-
H 2	Pseudomonas fluorescens	-	bacillus	+	-	-	-
H 38	Flexibacter sp.	-	filamentous bacillus	+	+	-	yellow

APPENDIX

TABLE 2. OXIDASE REACTION, CATALASE REACTION, DIFFUSIBLE PIGMENTS, O/F REACTIONS AND VIBRIOSTATIC AGENT SENSITIVITY OF SELECTED BACTERIA

Organism No.	Identification	(Cytochrome oxidase activity)		O/F Test	(pyocyanin production)		(fluorescens production)		Sensitivity to Vibriostatic Agent O/129
		OXIDASE	CATALASE	O	F	KINGS A	KINGS B		
H 3	Acinetobacter calcoaceticus	-	ND	+	-	ND	ND		ND
H 7	Acinetobacter sp	-	ND	+	-	ND	ND		ND
H 20	Acinetobacter sp	-	ND	+	-	ND	ND		ND
H 34	Acinetobacter sp	-	ND	-	-	ND	ND		ND
H 42	Acinetobacter calcoaceticus	-	ND	+	-	ND	ND		ND
H 22	Aeromonas hydrophila	+	ND	+	+	ND	ND		R
H 48	Aeromonas hydrophila	+	ND	+	+	MD	ND		R
H 31	Chromobacterium sp	+	ND	+	-	ND	ND		ND
H 16	Coryneform sp	-	+	ND	ND	ND	ND		ND
H 12	Enterobacter cloacae	-	ND	+	+	ND	ND		ND
H 15	Pseudomonas aeruginosa	+	ND	+	-	+	+		ND
H 2	Pseudomonas fluorescens	+	ND	+	-	-	+		ND
H 38	Flexibacter sp	+	-	+	-	ND	ND		ND

ND = not determined R = resistant

APPENDIX

TABLE 3. CARBOHYDRATE UTILIZATION AND POLYMER HYDROLYSIS BY SELECTED BACTERIA

Organism no.	Identification	Glucose(1,2)	Mannitol(1,2)	Maltose(1)	Ethanol(1)	Galactose(1)	Xylose(1)	Arabinose(1,2)	Lactose(1)	Inositol(1,2)	Sorbitol(2)	Rhamnose(2)	Sucrose(2)	Melibiose(2)	Amygdalin(2)	Cellobiose(1)	Cellulose(1)	Starch(1)	Gelatin(1)	Casein(1)	DNA agar(1)
H 3	Acinetobacter calcoaceticus	-	-	-	+	wk	-	-	-	-	-	-	-	-	-	-	-	-	-	NG	+
H 7	Acinetobacter sp	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	NG	-
H 20	Acinetobacter sp	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H 34	Acinetobacter sp	+	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
H 42	Acinetobacter calcoaceticus	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
H 22	Aeromonas hydrophila	+	+	-	wk	+	-	+	wk	-	-	-	+	-	+	-	-	+	+	-	+
H 48	Aeromonas hydrophila	+	+	-	-	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	+
H 31	Chromobacterium sp	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	wk	wk	-	-	-
H 16	Coryneform sp	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	+
H 12	Enterobacter cloacae	+	+	+	-	+	+	+	wk	-	+	+	+	+	+	+	-	wk	-	+	-
H 15	Pseudomonas aeruginosa	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-
H 2	Pseudomonas fluorescens	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	-	wk	+	+	-
H 38	Flexibacter sp	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	NG	-

NG - no growth

1) Utilization determined by Palleroni & Doudoroff basal medium for single carbon source and polymer hydrolase media

2) Utilization determined by API

APPENDIX

TABLE 4. UTILIZATION OF CITRATE, THIOSULPHATE, AMINO ACIDS, UREA AND PYRUVATE AND ENZYME ACTIVITIES BY SELECTED BACTERIA

Organism no	Identification	Acid/Neut/Alk																(1) Methods described in text (2) API method ND = not determined
		(1,2) ONPG B-galactosidase activity (1,2) ADH Arginine dehydrase (1,2) LDC Lysine decarbox- ylase (1,2) ODC Ornithine decarboxylase (2) Citrate Utilization (2) Sodium thiosulphate(H ₂ S) (1,2) Urease activity (2) TDA tryptophane deaminase activity (1,2) INDOLE tryptophanase activity (2) VP Pyruvate utilization (1) Phosphatase activity																
H 3	Acinetobacter calcoaceticus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 7	Acinetobacter sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 20	Acinetobacter sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 34	Acinetobacter sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 42	Acinetobacter calcoaceticus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 22	Aeromonas hydrophila	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND ND ND
H 48	Aeromonas hydrophila	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND ND ND
H 31	Chromobacterium sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
H 16	Coryneform sp	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND ND ND
H 12	Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 15	Pseudomonas aeruginosa	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND ND ND
H 2	Pseudomonas fluorescens	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 38	Flexibacter sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

APPENDIX

TABLE 5. SPECIFIC GROWTH RATES (μ) OF SELECTED ORGANISMS

Organism no	Identification	PYE M value	Time to Stationary Phase	Glucose minimal M
H 3	Acinetobacter calcoaceticus	0.54		ND
H 7	Acinetobacter sp	ND		ND
H 20	Acinetobacter sp	ND		ND
H 34	Acinetobacter sp	ND		ND
H 42	Acinetobacter calcoaceticus	ND		ND
H 22	Aeromonas hydrophila	0.37		ND
H 48	Aeromonas hydrophila	ND		ND
H 31	Chromobacterium sp	0.286	13 hrs	0.12
H 16	Coryneform sp	0.242		ND
H 12	Enterobacter sp	0.25	13 hrs	0.12
H 15	Pseudomonas aeruginosa	0.21	10 hrs	ND
H 2	Pseudomonas fluorescens	0.229	14 hrs	0.1
H 38	Flexibacter sp	0.222	16 hrs	0.06

ND = not determined

APPENDIX

TABLE 6. THE EFFECT OF CARBON/NITROGEN RATIO AND CARBON SOURCE IN BATCH CULTURE AND CONTINUOUS CULTURE ON THE CELL CHARACTERISTICS (BATCH CULTURE ONLY) AND ATTACHMENT OF PSEUDOMONAS FLUORESCENS

C/N Ratio and C source	Contact Angle (θ_s) on a lawn of cells from batch culture	60 mins attachment $A_{590} (x10^{-3})$ attached cells			
		BATCH CULTURE		CONTINUOUS CULTURE	
		PD	TCD	PD	TCD
Glucose Limited	8.5	27 (± 3) ^a	39 (± 5)	8 (± 2)	88 (± 8)
Nitrogen Limited	13.5	40 (± 2)	36 (± 4)	36 (± 24)	113 (± 10)
Glucose and Nitrogen XS	11.5	17 (± 2)	32 (± 3)	8 (± 2)	18 (± 3)
Glucose Nitrogen XS and Glycerol	9.5	54 (± 3)	38 (± 3)		
Lactose	9.0	12 (± 0.8)	18 (± 3)		
Galactose	8.5	12 (± 0.8)	20 (± 2)		
Mannose	11.5	24 (± 3)	23 (± 3)		
Sucrose	6.5	20 (± 2)	25 (± 0.4)		

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

APPENDIX

TABLE 7. THE EFFECT OF CARBON/NITROGEN RATIO AND CARBON SOURCE IN BATCH AND CONTINUOUS CULTURE ON THE CELL SURFACE CHARACTERISTICS (BATCH CULTURE ONLY) AND ATTACHMENT OF ENTEROBACTER CLOACAE

C/N Ratio and C Source	Contact Angle (θ_s) on lawns of cells from batch culture	(60 mins attachment)			
		$A_{590} (\times 10^{-3})$ attached cells			
		BATCH CULTURE		CONTINUOUS CULTURE	
		PD	TCD	PD	TCD
Glucose Limited	14.0	17 (± 2) ^a	27 (± 3)	3 (± 2)	21 (± 3)
Nitrogen Limited	15.0	6 (± 0.8)	21 (± 3)	27 (± 3)	39 (± 3)
Glucose & Nitrogen XS	11.5	7 (± 3)	33 (± 3)	6 (± 2)	30 (± 3)
Glucose, Nitrogen XS & Glycerol	11.5	7 (± 3)	33 (± 3)		
Lactose	NR	24 (± 3)	22 (± 3)		
Galactose	12.5	49 (± 4)	52 (± 3)		
Mannose	17.5	6 (± 0.8)	20 (± 2)		
Sucrose	15.0	24 (± 4)	20 (± 3)		

^a Parenthetical values are 95% confidence limits of the mean (n = 8)
NR = no result

APPENDIX

TABLE 8. THE EFFECT OF CARBON/NITROGEN RATIO AND CARBON SOURCE IN BATCH AND CONTINUOUS CULTURE ON THE CELL SURFACE CHARACTERISTICS (BATCH CULTURE ONLY) AND ATTACHMENT OF FLEXIBACTER SP.

C/N Ratio and C Source	Contact Angle (θ_s) on lawns of cells from batch culture	(60 min attachment)			
		$A_{590}(\times 10^{-3})$ attached cells			
		BATCH CULTURE		CONTINUOUS CULTURE	
		PD	TCD	PD	TCD
Glucose Limited	18.5	18 (± 3) ^a	27 (± 3)	18 (± 3)	31 (± 2)
Nitrogen Limited	18.0	21 (± 4)	32 (± 3)	7 (± 2)	18 (± 2)
Glucose and Nitrogen XS	17.0	14 (± 2)	24 (± 0.8)	11 (± 2)	16 (± 2)
Glucose, Nitrogen XS and Glycerol	16.0	15 (± 3)	23 (± 3)		
Lactose	8.0	8 (± 2)	16 (± 0.8)		
Galactose	13.0	13 (± 2)	19 (± 0.8)		
Mannose	8.0	12 (± 3)	20 (± 2)		
Sucrose	11.0	8 (± 2)	15 (± 0.8)		

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

APPENDIX

TABLE 9. THE EFFECT OF CARBON/NITROGEN RATIO IN CONTINUOUS CULTURE ON THE ATTACHMENT OF THE CHROMOBACTERIUM SP. (EXPERIMENT 2)

Carbon/Nitrogen Ratio	$A_{590}(\times 10^{-3})$ attached cells (60 min attachment)	
	PD	TCD
Glucose Limited	16 (± 2) ^a	47 (± 5)
Nitrogen Limited	22 (± 2)	34 (± 2)
Glucose and Nitrogen XS	32 (± 2)	49 (± 3)

^aParenthetical values are 95% confidence limits of the mean
(n = 8)

APPENDIX

TABLE 10. THE EFFECT OF GROWTH RATE IN CHEMOSTAT CULTURE ON THE ATTACHMENT OF PSEUDOMONAS FLUORESCENS TO POLYSTYRENE SURFACES (EXPERIMENT 2)

Dilution rate(h ⁻¹)	Attachmt time(mins)		A ₅₉₀ (x10 ⁻³) attached cells									
			5		15		30		45		60	
	PD	TCD	PD	TCD	PD	TCD	PD	TCD	PD	TCD	PD	TCD
0.05	9 (⁺ 3) ^a	30 (⁺ 3)	15 (⁺ 3)	28 (⁺ 3)	20 (⁺ 3)	36 (⁺ 3)	21 (⁺ 2)	37 (⁺ 2)	23 (⁺ 3)	35 (⁺ 0.8)		
0.1	8 (⁺ 2)	24 (⁺ 3)	12 (⁺ 3)	26 (⁺ 2)	11 (⁺ 3)	24 (⁺ 2)	11 (⁺ 3)	22 (⁺ 0.8)	11 (⁺ 4)	27 (⁺ 3)		
0.15	16 (⁺ 2)	40 (⁺ 7)	18 (⁺ 2)	29 (⁺ 5)	28 (⁺ 4)	40 (⁺ 3)	36 (⁺ 3)	41 (⁺ 5)	36 (⁺ 3)	27 (⁺ 4)		
0.2	9 (⁺ 3)	24 (⁺ 3)	6 (⁺ 3)	22 (⁺ 2)	11 (⁺ 3)	22 (⁺ 3)	13 (⁺ 5)	24 (⁺ 2)	18 (⁺ 3)	30 (⁺ 3)		

^aParenthetical values are 95% confidence limits of the mean (n = 8)

APPENDIX

TABLE 11. THE EFFECT OF GROWTH RATE IN CHEMOSTAT CULTURE ON THE ATTACHMENT OF ENTEROBACTER CLOACAE TO POLYSTYRENE SURFACES (EXPERIMENT 2)

Dilution rate(h ⁻¹)	Attchmt time(mins)		A ₅₉₀ (x10 ⁻³) attached cells									
			5		15		30		45		60	
	PD	TCD	PD	TCD	PD	TCD	PD	TCD	PD	TCD		
0.05	9 (⁺ 3) ^a	20 (⁺ 3)	9 (⁺ 2)	15 (⁺ 3)	6 (⁺ 0.8)	15 (⁺ 3)	5 (⁺ 2)	20 (⁺ 2)	10 (⁺ 3)	26 (⁺ 3)		
0.1	6 (⁺ 2)	21 (⁺ 3)	7 (⁺ 2)	18 (⁺ 2)	9 (⁺ 2)	19 (⁺ 3)	15 (⁺ 3)	23 (⁺ 2)	14 (⁺ 2)	25 (⁺ 2)		
0.15	4 (⁺ 0.8)	20 (⁺ 0.8)	8 (⁺ 3)	19 (⁺ 3)	7 (⁺ 3)	22 (⁺ 3)	6 (⁺ 0.8)	20 (⁺ 4)	9 (⁺ 3)	19 (⁺ 3)		
0.2	4 (⁺ 2)	15 (⁺ 0.8)	4 (⁺ 2)	20 (⁺ 2)	8 (⁺ 2)	18 (⁺ 2)	11 (⁺ 2)	21 (⁺ 3)	13 (⁺ 3)	20 (⁺ 2)		

^aParenthetical values are 95% confidence limits of the mean (n = 8)

APPENDIX

TABLE 12. THE EFFECT OF GROWTH RATE IN CHEMOSTAT CULTURES ON THE ATTACHMENT OF THE CHROMOBACTERIUM SP TO POLYSTYRENE SURFACES (EXPERIMENT 2)

Dilution rate(h ⁻¹)	Attchmt time(mins)	A ₅₉₀ (x10 ⁻³) attached cells									
		5		15		30		45		60	
		PD	TCD	PD	TCD	PD	TCD	PD	TCD	PD	TCD
0.05		46 (⁺ 3) ^a	58 (⁺ 6)	70 (⁺ 5)	78 (⁺ 3)	96 (⁺ 5)	93 (⁺ 4)	122 (⁺ 8)	96 (⁺ 4)	106 (⁺ 7)	88 (⁺ 4)
0.1		50 (⁺ 4)	57 (⁺ 5)	118 (⁺ 10)	91 (⁺ 7)	160 (⁺ 18)	93 (⁺ 3)	193 (⁺ 15)	82 (⁺ 8)	205 (⁺ 8)	100 (⁺ 10)
0.15		115 (⁺ 7)	92 (⁺ 5)	145 (⁺ 10)	110 (⁺ 5)	192 (⁺ 16)	103 (⁺ 10)	199 (⁺ 13)	116 (⁺ 14)	196 (⁺ 13)	113 (⁺ 3)
0.2		54 (⁺ 4)	54 (⁺ 6)	63 (⁺ 8)	44 (⁺ 3)	42 (⁺ 8)	54 (⁺ 3)	49 (⁺ 8)	52 (⁺ 3)	37 (⁺ 3)	39 (⁺ 3)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

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TABLE 13. THE EFFECT OF GROWTH RATE IN CHEMOSTAT CULTURE ON THE ATTACHMENT OF THE FLEXIBACTER SP. TO POLYSTYRENE SURFACES (EXPERIMENT 2)

Dilution rate (h ⁻¹)	Attchmt time(mins)	A ₅₉₀ (x10 ⁻³) attached cells									
		5		15		30		45		60	
		PD	TCD	PD	TCD	PD	TCD	PD	TCD	PD	TCD
0.05		6 (⁺ 3) ^a	23 (⁺ 3)	7 (⁺ 2)	19 (⁺ 2)	13 (⁺ 2)	26 (⁺ 0.8)	16 (⁺ 3)	33 (⁺ 4)	16 (⁺ 3)	33 (⁺ 4)
0.1		10 (⁺ 3)	22 (⁺ 3)	14 (⁺ 3)	29 (⁺ 3)	22 (⁺ 3)	38 (⁺ 5)	30 (⁺ 2)	41 (⁺ 7)	32 (⁺ 5)	45 (⁺ 3)
0.15		14 (⁺ 3)	29 (⁺ 3)	67 (⁺ 6)	49 (⁺ 4)	156 (⁺ 18)	109 (⁺ 20)	201 (⁺ 26)	149 (⁺ 8)	211 (⁺ 13)	164 (⁺ 19)
0.2		34 (⁺ 6)	32 (⁺ 3)	86 (⁺ 10)	59 (⁺ 7)	142 (⁺ 10)	99 (⁺ 10)	195 (⁺ 7)	141 (⁺ 20)	254 (⁺ 12)	195 (⁺ 25)

^a Parenthetical values are 95% confidence limits of the mean (n = 8)

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TABLE 14. THE EFFECT OF ELECTRICAL DOUBLE-LAYER THICKNESS (1/K) ON THE ATTACHMENT OF BACTERIA TO SURFACES

	Pseudomonas fluorescens		Enterobacter cloacae		Chromobacterium sp		Flexibacter sp	
1/K(nm)	PD	TCD	PD	$A_{590}(\times 10^{-3})$ TCD	PD	TCD	PD	TCD
Approa- ching Infinity	16(± 3) ^a	30(± 3)	80(± 8)	94(± 3)	51(± 5)	41(± 7)	155(± 13)	122(± 10)
3.1	34(± 0.8)	45(± 6)	98(± 6)	92(± 8)	31(± 3)	36(± 4)	140(± 18)	64(± 20)
1.5	25(± 3)	39(± 6)	74(± 13)	66(± 5)	14(± 2)	26(± 3)	40(± 3)	37(± 3)
1.0	33(± 4)	39(± 4)	71(± 8)	62(± 5)	46(± 3)	46(± 3)	28(± 0.8)	25(± 3)
0.5	23(± 3)	29(± 6)	69(± 9)	78(± 3)	10(± 8)	28(± 3)	32(± 3)	34(± 2)
0.3	15(± 0.8)	17(± 2)	27(± 0.8)	20(± 5)	22(± 2)	22(± 2)	142(± 13)	101(± 12)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 15. THE EFFECT OF LIQUID SURFACE TENSION ON THE ATTACHMENT OF BACTERIA TO SURFACES

Liquid Surface Tension γ_{LV} ($\mu J/cm^2$)	Pseudomonas fluorescens		Enterobacter cloacae		Chromobacterium sp		Flexibacter sp	
	$A_{590}(\times 10^{-3})$ of attached cells							
	PD	TCD	PD	TCD	PD	TCD	PD	TCD
7.2	24(± 2) ^a	24(± 2)	98(± 9)	81(± 4)	180(± 25)	126(± 12)	24(± 0.8)	23(± 3)
7.1	18(± 3)	42(± 5)	68(± 9)	76(± 3)	117(± 16)	129(± 21)	21(± 3)	24(± 3)
6.9	20(± 3)	35(± 2)	74(± 8)	79(± 3)	125(± 23)	134(± 19)	21(± 2)	20(± 2)
6.8	19(± 3)	33(± 3)	48(± 2)	52(± 6)	108(± 20)	133(± 16)	18(± 2)	17(± 3)
6.7	20(± 2)	30(± 0.8)	34(± 2)	49(± 2)	150(± 10)	134(± 14)	18(± 3)	17(± 2)
6.6	14(± 4)	23(± 3)	26(± 3)	43(± 2)	99(± 7)	101(± 23)	13(± 2)	19(± 4)
6.5	6(± 2)	19(± 2)	30(± 2)	40(± 3)	48(± 8)	84(± 5)	9(± 3)	18(± 3)
Saline Contact(θ_s) angle of cells($^{\circ}$)	14		10		12		13	
Solid Surface ^b Tension of cells ($\mu J/cm^2$)	6.99		7.09		7.05		7.02	

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

^b Calculated using the Equation of State Approach (Neumann et al, 1974)

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TABLE 16. THE DETACHMENT OF BACTERIA FROM SOLID SURFACES BY CHEMICAL AND pH TREATMENTS

	Pseudomonas fluorescens		Enterobacter cloacae		Chromobacterium sp		Flexibacter sp	
Treatment	PD	TCD	A ₅₉₀ (x10 ⁻³) attached cells		PD	TCD	PD	TCD
			PD	TCD				
Control(no detachment)	60(⁺¹⁰) ^a	73(⁺⁸)	43(⁺²)	40(⁺⁴)	118(⁺¹⁶)	19(⁺⁴)	16(⁺²)	14(^{+0.8})
0.1M NaCl	68(⁺⁸)	63(⁺⁸)	38(⁺³)	35(⁺⁴)	88(⁺¹¹)	20(⁺³)	17(⁺²)	16(^{+0.8})
0.1M MgCl ₂	62(⁺⁶)	65(⁺⁵)	34(⁺⁷)	28(⁺⁴)	100(⁺¹⁰)	18(^{+0.8})	13(^{+0.8})	15(⁺²)
0.1%(v/v)SLS	4(0)	20(⁺⁴)	1(^{+0.8})	16(⁺⁴)	6(⁺²)	18(⁺²)	3(^{+0.8})	11(^{+0.8})
0.1%(v/v)RBS	21(⁺⁵)	41(⁺⁸)	39(⁺⁴)	39(⁺³)	22(⁺³)	22(^{+0.8})	6(⁺²)	13(⁺²)
0.1%(v/v)Tween-80	22(⁺³)	42(⁺⁵)	20(⁺⁴)	27(⁺³)	19(⁺³)	17(⁺²)	15(⁺³)	15(^{+0.8})
pH 5	68(⁺⁸)	70(⁺⁹)	46(⁺⁵)	40(⁺³)	90(⁺¹⁰)	14(⁺³)	18(⁺²)	18(⁺²)
pH 7	74(⁺⁵)	75(⁺⁴)	51(⁺³)	44(⁺⁷)	89(⁺¹⁰)	19(⁺²)	32(⁺³)	23(^{+0.8})
pH 9	65(⁺¹⁰)	77(⁺⁹)	39(⁺⁴)	44(⁺⁵)	80(⁺⁷)	14(⁺²)	14(⁺²)	19(⁺³)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 17. THE EFFECT OF THE PRE-TREATMENT OF PD AND TCD SURFACES WITH CARBOHYDRATES ON THE ATTACHMENT OF PSEUDOMONAS FLUORESCENS

Carbohydrate	$A_{590}(\times 10^{-3})$ of attached cells	
	PD	TCD
Control	214 (± 27) ^a	36 (± 4)
Glucose	408 (± 27)	51 (± 8)
Mannitol	304 (± 19)	51 (± 8)
Maltose	267 (± 25)	36 (± 8)
Inositol	290 (± 30)	49 (± 3)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 18. THE EFFECT OF THE POST-TREATMENT OF PD AND TCD SURFACES WITH CARBOHYDRATES ON THE ATTACHMENT OF PSEUDOMONAS FLUORESCENS

Carbohydrate	$A_{590}(\times 10^{-3})$ of attached cells	
	PD	TCD
Control	316 (± 23)	44 (± 9)
Glucose	341 (± 42)	45 (± 5)
Mannitol	334 (± 34)	40 (± 4)
Maltose	308 (± 25)	23 (± 3)
Inositol	283 (± 34)	51 (± 8)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 19. THE EFFECT OF GLUCOSE CONCENTRATION ON THE ATTACHMENT OF *PSEUDOMONAS FLUORESCENS* AFTER GROWTH IN CONTINUOUS CULTURE ($D = 0.025h^{-1}$) IN GLUCOSE LIMITED, NITROGEN LIMITED AND GLUCOSE/NITROGEN SUFFICIENT CONDITIONS

Glucose mg/ml	Carbon Limited Culture		Nitrogen Limited Culture		Carbon/Nitrogen Sufficient Culture	
	PD	$A_{590}(x10^{-3})$ TCD	PD	TCD	PD	TCD
0	8 (± 2) ^a	88 (± 8)	36 (± 22)	113 (± 10)	8 (± 2)	18 (± 3)
0.5	59 (± 23)	144 (± 13)	33 (± 22)	99 (± 8)	18 (± 3)	21 (± 3)
2	93 (± 37)	115 (± 18)	27 (± 3)	76 (± 10)	6 (± 3)	19 (± 3)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 20. THE EFFECT OF GLUCOSE CONCENTRATION ON THE ATTACHMENT OF *ENTEROBACTER CLOACAE* AFTER GROWTH IN CONTINUOUS CULTURE ($D = 0.025h^{-1}$) IN GLUCOSE LIMITED, NITROGEN LIMITED AND GLUCOSE/NITROGEN SUFFICIENT CONDITIONS

Glucose mg/ml	Carbon Limited Culture		Nitrogen Limited Culture		Carbon/Nitrogen Sufficient Culture	
	PD	$A_{590}(x10^{-3})$ TCD	PD	TCD	PD	TCD
0	3 (± 2) ^a	21 (± 3)	27 (± 3)	39 (± 3)	6 (± 3)	30 (± 4)
0.5	7 (± 2)	30 (± 2)	8 (± 2)	36 (± 7)	4 (± 2)	33 (± 3)
2	7 (± 0.8)	26 (± 2)	10 (± 3)	35 (± 3)	3 (± 0.8)	30 (± 6)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 21. THE EFFECT OF GLUCOSE CONCENTRATION ON THE ATTACHMENT OF THE CHROMOBACTERIUM SP AFTER GROWTH IN CONTINUOUS CULTURE ($D = 0.025h^{-1}$) IN GLUCOSE LIMITED, NITROGEN LIMITED AND GLUCOSE/NITROGEN SUFFICIENT CONDITIONS

Glucose mg/ml	Carbon Limited Culture		Nitrogen Limited Culture		Carbon/Nitrogen Sufficient Culture	
	PD	$A_{590} (x10^{-3})$ TCD	PD	TCD	PD	TCD
0	16 (± 3) ^a	47 (± 3)	22 (± 3)	34 (± 3)	32 (± 3)	49 (± 3)
0.5	106 (± 20)	100 (± 3)	18 (± 2)	29 (± 3)	28 (± 4)	41 (± 4)
2	114 (± 5)	95 (± 4)	30 (± 3)	37 (± 4)	26 (± 2)	35 (± 3)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 22. THE EFFECT OF GLUCOSE CONCENTRATION ON THE ATTACHMENT OF THE FLEXIBACTER SP AFTER GROWTH IN CONTINUOUS CULTURE ($D = 0.025h^{-1}$) IN GLUCOSE LIMITED, NITROGEN LIMITED AND GLUCOSE/NITROGEN SUFFICIENT CONDITIONS

Glucose mg/ml	Carbon Limited Culture		Nitrogen Limited Culture		Carbon/Nitrogen Sufficient Culture	
	PD	$A_{590} (x10^{-3})$ TCD	PD	TCD	PD	TCD
0	18 (± 3) ^a	31 (± 2)	7 (± 3)	18 (± 3)	11 (± 3)	16 (± 3)
0.5	10 (± 3)	23 (± 2)	6 (± 3)	17 (± 3)	13 (± 3)	19 (± 0.8)
2	6 (± 0.8)	17 (± 2)	7 (± 2)	17 (± 3)	10 (± 4)	20 (± 2)

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 23. THE EFFECT OF GROWTH PHASE IN BATCH CULTURE ON SUBSEQUENT BACTERIAL ATTACHMENT

Species	Growth Phase	$A_{590}(\times 10^{-3})$ attached cells			
		PD	TCD	PD I_a	TCD I_a
<u>Pseudomonas fluorescens</u>	Exponential	13(± 2) ^a	22(± 3)	1	1
	Stationary	13(± 2)	34(± 5)	1	1
	Death	14(0)	22(± 3)	1	1
<u>Enterobacter cloacae</u>	Exponential	18(± 2)	27(± 2)	1	1
	Stationary	42(± 17)	29(± 5)	2.3	1
	Death	60(± 4)	27(± 0.8)	3.3	1
<u>Chromobacterium</u> sp	Exponential	35(± 4)	21(± 3)	1	1
	Stationary	45(± 4)	16(± 2)	1.3	1
	Death	46(± 3)	24(± 3)	1.3	1
<u>Flexibacter</u> sp	Exponential	62(± 17)	37(± 8)	1	1
	Stationary	96(± 7)	44(± 3)	1.5	1.2
	Death	22(± 2)	22(± 2)	0.39	0.59

^a Parenthetical values represent 95% confidence limits of the mean (n=8)

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TABLE 24. THE EFFECT OF CHLORAMPHENICOL, SODIUM PERIODATE AND PROTEASE ON BACTERIAL DETACHMENT

Species	Treatment	$A_{590}(\times 10^{-3})$ attached cells			
		PD	TCD	PD I_d	TCD I_d
<u>Pseudomonas fluorescens</u>	Control(no detachment)	27(± 3) ^a	38(± 2)	1	1
	Chloramphenicol (5 μ g/ml)	23(± 2)	36(± 3)	1	1
	Sodium periodate(1% w/v)	25(± 3)	35(± 3)	1	1
	Protease (1 unit/5 ml)	5(± 2)	14(± 2)	0.19	0.36
<u>Enterobacter cloacae</u>	Control(no detachment)	30(± 0.8)	19(± 3)	1	1
	Chloramphenicol (5 μ g/ml)	36(± 3)	22(± 3)	1.2	1
	Sodium periodate(1% w/v)	35(± 2)	19(± 3)	1.2	1
	Protease (1 unit/5 ml)	4(± 0.8)	13(± 2)	0.13	0.68
<u>Chromobacterium</u> sp	Control(no detachment)	80(± 7)	20(± 2)	1	1
	Chloramphenicol (5 μ g/ml)	86(± 6)	21(± 0.8)	1	1
	Sodium periodate(1% w/v)	98(± 10)	19(± 0.8)	1.2	1
	Protease (1 unit/5 ml)	58(± 3)	16(± 2)	0.73	1
<u>Flexibacter</u> sp	Control(no detachment)	147(± 0.8)	48(± 3)	1	1
	Chloramphenicol (5 μ g/ml)	139(± 12)	49(± 8)	1	1
	Sodium periodate(1% w/v)	137(± 26)	42(± 5)	1	1
	Protease (1 unit/5 ml)	135(± 16)	44(± 7)	1	1

^a Parenthetical values represent 95% confidence limits of the mean (n = 8)

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TABLE 25. THE EFFECT OF TEMPERATURE ON BACTERIAL ATTACHMENT TO PD AND TCD SURFACES

Temp (°C)	$A_{590}(\times 10^{-3})$ attached cells							
	<i>Pseudomonas fluorescens</i>		<i>Enterobacter cloacae</i>		<i>Chromobacterium</i> sp		<i>Flexibacter</i> sp	
	PD	TCD	PD	TCD	PD	TCD	PD	TCD
4	33(⁺ 3) ^a	61(⁺ 3)	141(⁺ 0.8)	31(⁺ 0.8)	56(⁺ 9)	48(⁺ 3)	285(⁺ 33)	149(⁺ 36)
15	42(⁺ 8)	60(⁺ 7)	152(⁺ 6)	32(⁺ 3)	68(⁺ 12)	65(⁺ 6)	292(⁺ 15)	184(⁺ 21)
20	42(⁺ 10)	70(⁺ 5)	186(⁺ 13)	47(⁺ 3)	104(⁺ 9)	120(⁺ 7)	232(⁺ 18)	166(⁺ 11)
25	51(⁺ 10)	77(⁺ 5)	179(⁺ 5)	28(⁺ 2)	51(⁺ 10)	120(⁺ 8)	199(⁺ 18)	142(⁺ 18)
30	36(⁺ 4)	48(⁺ 4)	149(⁺ 17)	26(⁺ 3)	16(⁺ 2)	71(⁺ 20)	193(⁺ 10)	146(⁺ 7)
37	23(⁺ 3)	35(⁺ 4)	137(⁺ 8)	28(⁺ 2)	10(⁺ 2)	73(⁺ 3)	178(⁺ 25)	158(⁺ 18)
45	13(⁺ 8)	18(⁺ 3)	139(⁺ 5)	28(⁺ 2)	10(⁺ 3)	29(⁺ 3)	103(⁺ 11)	128(⁺ 16)

^aParenthetical values are 95% confidence limits of the mean (n = 8)

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TABLE 26. THE EFFECT OF pH ON BACTERIAL ATTACHMENT TO PD AND TCD SURFACES

pH	$A_{590}(\times 10^{-3})$ attached cells							
	Pseudomonas fluorescens PD	Pseudomonas fluorescens TCD	Enterobacter cloacae PD	Enterobacter cloacae TCD	Chromobacterium sp PD	Chromobacterium sp TCD	Flexibacter sp PD	Flexibacter sp TCD
4.0	10(± 2) ^a	28(± 5)	17(± 3)	13(± 3)	10(± 4)	23(± 6)	25(± 0.8)	28(± 3)
4.5	11(± 0.8)	24(± 4)	18(± 3)	11(± 3)	11(± 3)	16(± 0.8)	22(± 4)	22(± 2)
5.0	7(± 2)	22(± 2)	17(± 3)	28(± 3)	7(± 3)	14(± 0.8)	17(± 0.8)	22(± 3)
5.5	13(± 0.8)	22(± 2)	15(± 3)	22(± 3)	87(± 19)	15(± 2)	15(± 0.8)	15(± 2)
6.0	11(± 9)	28(± 4)	17(± 3)	22(± 3)	123(± 19)	28(± 6)	19(± 2)	14(± 7)
6.5	33(± 3)	75(± 10)	16(± 2)	28(± 2)	45(± 8)	16(± 4)	12(± 7)	11(± 3)
7.0	36(± 4)	70(± 8)	24(± 2)	39(± 2)	19(± 10)	12(± 2)	13(± 2)	13(± 3)
7.5	18(± 5)	26(± 2)	19(± 2)	21(± 0.8)	9(± 4)	11(± 4)	10(± 3)	12(± 0.8)
8.0	17(± 3)	28(± 3)	19(± 2)	24(± 2)	37(± 28)	7(± 10)	9(± 2)	11(± 2)
8.5	5(± 2)	14(± 0.8)	14(± 0.8)	17(± 2)	39(± 42)	14(± 2)	11(± 0.8)	10(± 0.8)
9.0	6(± 2)	14(± 3)	14(± 0.8)	16(± 2)	4(± 2)	11(± 3)	5(± 2)	11(± 0.8)
9.5	3(± 2)	9(± 0.8)	7(± 2)	11(± 3)	5×10^{-4} (± 0.8)	7(± 3)	4(± 0.8)	10(± 3)

^aParenthetical values are 95% confidence limits of the mean (n = 8)